

**This Page Is Inserted by IFW Operations
and is not a part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- **BLACK BORDERS**
- **TEXT CUT OFF AT TOP, BOTTOM OR SIDES**
- **FADED TEXT**
- **ILLEGIBLE TEXT**
- **SKEWED/SLANTED IMAGES**
- **COLORED PHOTOS**
- **BLACK OR VERY BLACK AND WHITE DARK PHOTOS**
- **GRAY SCALE DOCUMENTS**

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

THIS PAGE BLANK (USPTO)

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 47/48

A1

(11) International Publication Number:

WO 98/03204

(43) International Publication Date: 29 January 1998 (29.01.98)

(21) International Application Number: PCT/US96/12124
(22) International Filing Date: 23 July 1996 (23.07.96)

(71) Applicant: OREGON HEALTH SCIENCES UNIVERSITY
[US/US]; 3181 S.W. Sam Jackson Park Road, Portland, OR
97201-3098 (US).

(72) Inventors: YATVIN, Milton, B.; 5226 S.W. Northwood
Avenue, Portland, OR 97201 (US). STOWELL, Michael,
H., B.; Catalina 438 #105, Pasadena, CA 91106 (US).

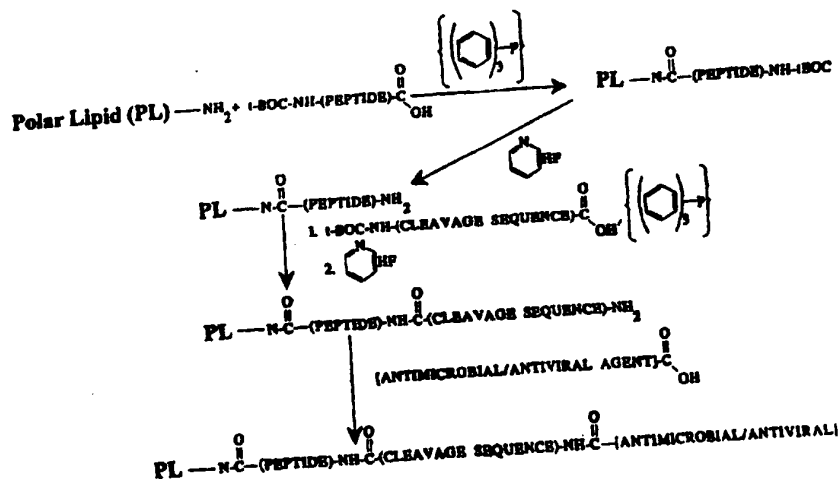
(74) Agent: NOONAN, Kevin, E.; McDonnell Boenken Hulbert &
Berghoff, 7th floor, 300 South Wacker Drive, Chicago, IL
60606 (US).

(81) Designated States: AU, CA, JP, KR, European patent (AT,
BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC,
NL, PT, SE).

Published

With international search report.

(54) Title: COVALENT POLAR LIPID CONJUGATES WITH BIOLOGICALLY-ACTIVE COMPOUNDS FOR USE IN SALVES



(57) Abstract

This invention herein describes a method of facilitating the entry of drugs into cells and tissues at pharmacokinetically useful levels and also a method of targeting drugs to specific organelles within the cell. This polar lipid/drug conjugate targeting invention embodies an advance over other drug targeting methods because through this method, intracellular drug concentrations may reach levels which are orders of magnitude higher than those achieved otherwise. Furthermore, it refines the drug delivery process by allowing therapeutic agents to be directed to certain intracellular structures. This technology is appropriate for use with antiproliferative, antibiotic, antimycotic, antiviral and antineoplastic drugs, in particular in combination with a multiplicity of other emollients and agents to make up topically-active substances such as salves, for rapid and efficient introduction of such agents through the epidermis for treatment of skin diseases and other disorders.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

COVALENT POLAR LIPID CONJUGATES WITH BIOLOGICALLY-ACTIVE COMPOUNDS FOR USE IN SALVES

BACKGROUND OF THE INVENTION

5

1. Field of the Invention

A major goal in the pharmacological arts has been the development of methods and compositions to facilitate the specific delivery of therapeutic and other agents to the appropriate cells and tissues that would benefit from such treatment, and the avoidance of the general physiological effects of the inappropriate delivery of such agents to other cells or tissues of the body. One common example of the need for such specificity is in the field of antiproliferative agent therapy for the treatment of skin diseases and disorders, in which the amount of a variety of antiproliferative agents to be safely administered topically or locally to a patient is limited by their systemic cytotoxic effects.

10

In addition, it is recognized in the medical arts that certain subcellular organelles are the sites of pharmacological action of certain drugs or are involved in the biological response to certain stimuli. Specific delivery of diagnostic or therapeutic compounds to such intracellular organelles is thus desirable to increase the specificity and effectiveness of such clinical diagnostic or therapeutic techniques. The invention also provides polar lipid drug conjugates that target dermal, intradermal and infradermal structures in skin for delivery of therapeutic agents for the treatment of skin diseases and disorders.

20

25

A. Drug Targeting

30

It is desirable to increase the efficiency and specificity of administration of a therapeutic agent to the cells of the relevant tissues in a variety of pathological states. This is particularly important as relates to antiproliferative agents. Such agents typically have pleiotropic antibiotic and cytotoxic effects that damage or destroy uninvolved cells and tissues as well as cells and tissues comprising the pathological site. Thus, an efficient delivery system which would enable the delivery of such drugs specifically to the diseased or affected tissues cells would increase the efficacy of treatment and reduce the associated "side effects" of such

drug treatments, and also serve to reduce morbidity and mortality associated with clinical administration of such drugs.

5 Numerous methods for enhancing the biological activity and the specificity of drug action have been proposed or attempted. To date, however, efficient or specific drug delivery remains to be predictably achieved.

10 An additional challenge in designing an appropriate drug delivery scheme is to include within the drug conjugate a functionality which could either accelerate or reduce the rate at which the drug is released upon arrival at the desired site. Such a functionality would be especially valuable if it allowed differential rates of drug release.

15 Medicinal salves and ointments for topical treatment purposes are known in the prior art for the treatment of a variety of pathological conditions. A multitude of pathological and other conditions have been treated by topical application of many classes of compounds in a variety of carriers, such as salves and ointments. However, carriers used in these conventional treatments are in no way specific for deposition of drugs, and suffer from non-specific deposition of the antiproliferative drug into both healthy and affected portions of the skin. Appropriate concentrations of topically-applied antiproliferative drugs, for example, are currently limited by the escape of the active agent(s) into the systemic circulation, with deleterious effects on other tissues and organs. An example of such a situation is the use of the drug methotrexate to treat psoriasis, where the amount of methotrexate that is capable of being topically applied is limited by hepato- and nephrotoxicity caused by systemic escape of the compound from the skin.

20 There remains a need in the art for an effective means for delivering biologically-active compounds, specifically drugs including antiproliferative drugs, to skin by topical administration of salves, ointments, and the like. Advantageous embodiments of such delivery means are formulated to efficiently deliver the biologically-active compound to the appropriate layer of the skin, while minimizing transit of the compound into the systemic circulation.

30

BRIEF SUMMARY OF THE INVENTION

The present invention is directed to an improved method for delivering biologically-active compounds, particularly drugs including preferably antiproliferative, antibiotic, antimycotic, antiviral and antineoplastic drugs, to cells comprising skin in animals *in vivo* and *in vitro*. This delivery system achieves specific delivery of such biologically-active compounds through conjugating the compounds with a polar lipid carrier. This invention has the specific advantage of facilitating the entry of such compounds into cells *via* a polar lipid carrier, achieving effective intracellular concentration of such compounds more efficiently and with more specificity than conventional delivery systems. The invention particularly provides pharmaceutical composition comprising the drug/polar lipid conjugates of the invention formulated with a medicinal ointment or salve for treatment of a variety of skin disorders.

The invention provides compositions of matter comprising a biologically-active compound covalently linked to a polar lipid carrier molecule. Preferred embodiments also comprise a spacer molecule having two linker functional groups, wherein the spacer has a first end and a second end and wherein the lipid is attached to the first end of the spacer through a first linker functional group and the biologically-active compound is attached to the second end of the spacer through a second linker functional group. In preferred embodiments, the biologically-active compound is a drug, most preferably an antiproliferative drug or agent, an antibiotic drug, an antiviral drug, an antineoplastic drug or a corticosteroid. Preferred polar lipids include but are not limited to acyl- and acylated carnitine, sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid. Preferred biologically-active compounds include antineoplastic and antiproliferative agents such as methotrexate, corticosteroids, antimycotics, antibiotics and antiviral compounds. Pharmaceutical compositions comprising the drug/polar lipid conjugates of the invention formulated with a medicinal ointment or salve are also provided.

The invention also provides compositions of matter comprising a biologically-active compound covalently linked to a lipid, most preferably a polar

lipid, carrier molecule *via* a spacer molecule wherein the spacer allows the biologically-active compound to act without being released at an intracellular site. In these embodiments of the invention, the first linker functional group attached to the first end of the spacer is characterized as "strong" and the second linker functional group attached to the second end of the spacer is characterized as "weak", with reference to the propensity of the covalent bonds between each end of the spacer molecule to be broken.

In other embodiments of the compositions of matter of the invention, the spacer allows the facilitated hydrolytic release of the biologically-active compound at an intracellular site. Other embodiments of the spacer facilitate the enzymatic release of the biologically-active compound at an intracellular site. In particularly preferred embodiments, the spacer functional group is hydrolyzed by an enzymatic activity found in skin, preferably an esterase and most preferably an esterase having a differential expression and activity profile in different skin layers. In additional preferred embodiments, specific release of biologically-active compounds is achieved by enzymatic or chemical release of the biologically-active compound by intracellular cleavage of a cleavable linker moiety in cells infected by a pathogenic organism or otherwise expressing a disease state (*for example*, hyperplasia associated with a benign or malignant skin condition) *via* an enzymatic activity specific for such a pathogenic organism or disease state, or by extracellular cleavage of a cleavable linker moiety *via* an enzymatic activity specific for a pathogenic organism or disease state.

The invention also provides polar lipid drug conjugates that target dermal, intradermal and infradermal structures in skin for delivery of therapeutic agents for the treatment of skin diseases and disorders. Specifically, the invention provides such conjugates comprising a spacer that allows facilitated hydrolytic or enzymatic release of the biologically-active compound at a dermal, intradermal or infradermal site in skin.

In another embodiment of this aspect of the invention, the spacer molecule is a peptide of formula (amino acid)_n, wherein n is an integer between 2 and 25, preferably wherein the peptide comprises a polymer of one or more amino acids.

In other embodiments of the compositions of matter of the invention, the biologically-active compound of the invention has a first functional linker group, and a lipid, most preferably a polar lipid, carrier has a second functional linker group, and the compound is covalently linked directly to the lipid carrier by a chemical bond between the first and second functional linker groups. In preferred
5 embodiments, each of the first and second functional linker groups is a hydroxyl group, a primary or secondary amino group, a phosphate group or substituted derivatives thereof or a carboxylic acid group.

In another aspect of the invention is provided compositions of matter comprising a drug, most preferably an antiproliferative drug, an antineoplastic drug,
10 an antibiotic, an antimycotic, or an antiviral drug, covalently linked to a polar lipid carrier molecule. Preferred embodiments also comprise a spacer molecule having two linker functional groups, wherein the spacer has a first end and a second end and wherein the lipid is attached to the first end of the spacer through a first linker
15 functional group and the drug is attached to the second end of the spacer through a second linker functional group. Preferred embodiments of the invention are provided wherein the drug is an antiproliferative agent, such as methotrexate, an antiviral agent such as an antiherpetic agent, an antibiotic agent such as rifampicin or streptomycin, or an antimycotic such as econazole. Preferred polar lipids include
20 but are not limited to acyl- and acylated carnitine, sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid. Pharmaceutical compositions comprising the drug/polar lipid conjugates of the invention formulated with a medicinal ointment or salve are also provided.

25 The invention also provides compositions of matter comprising an antiproliferative agent, an antineoplastic drug, an antibiotic, an antimycotic, or an antiviral drug, covalently linked to a polar lipid carrier molecule *via* a spacer molecule, wherein the spacer allows the drug to act without being released at an intracellular site. In these embodiments of the invention, the first linker functional
30 group attached to the first end of the spacer is characterized as "strong" and the second linker functional group attached to the second end of the spacer is

characterized as "weak", with reference to the propensity of the covalent bonds between each end of the spacer molecule to be broken.

5 In other embodiments of the compositions of matter of the invention, the spacer allows the facilitated hydrolytic release of an antiproliferative drug, an antineoplastic drug, an antibiotic, an antimycotic, or an antiviral drug, at an intracellular site. Other embodiments of the spacer facilitate the enzymatic release of the antiproliferative, antineoplastic, antibiotic, antimycotic or antiviral drugs of the invention at an intracellular site. In particularly preferred embodiments, the spacer functional group is hydrolyzed by an enzymatic activity found in skin, preferably an esterase and most preferably an esterase having a differential expression and activity profile in different skin layers. In additional preferred 10 embodiments, specific release of the antiproliferative, antineoplastic, antibiotic, antimycotic or antiviral drugs of the invention is achieved by enzymatic or chemical release of these drugs by intracellular cleavage of a cleavable linker moiety in cells infected by a pathogenic organism or otherwise expressing a disease state (*for example*, hyperplasia associated with a benign or malignant skin condition) *via* an enzymatic activity specific for such a pathogenic organism or disease state, or by extracellular cleavage of a cleavable linker moiety *via* an enzymatic activity specific for a pathogenic organism or disease state.

20 The invention also provides polar lipid conjugates of of the antiproliferative, antineoplastic, antibiotic, antimycotic or an antiviral drugs of the invention that target dermal, intradermal and infradermal structures in skin for delivery of therapeutic agents for the treatment of skin diseases and disorders. Specifically, the invention provides such conjugates comprising a spacer that allows facilitated 25 hydrolytic or enzymatic release of the of such antiproliferative, antineoplastic, antibiotic, antimycotic or an antiviral drugs at a dermal, intradermal or infradermal site in skin.

30 In another embodiment of this aspect of the invention, the spacer molecule is a peptide of formula (amino acid)_n, wherein n is an integer between 2 and 25, preferably wherein the peptide comprises a polymer of one or more amino acids.

In still further embodiments of the compositions of matter of the invention are provided an antiproliferative drug, an antineoplastic drug, an antibiotic, an antimycotic, or an antiviral drug, having a first functional linker group, and a polar lipid carrier having a second functional linker group, wherein the drug is covalently linked directly to the polar lipid carrier by a chemical bond between the first and second functional linker groups. In preferred embodiments, each of the first and second functional linker groups is a hydroxyl group, a primary or secondary amino group, a phosphate group or substituted derivatives thereof or a carboxylic acid group. Preferred embodiments of the invention are provided wherein the drug is an antiproliferative agent, such as methotrexate, an antiviral agent such as an antiherpetic agent, an antibiotic agent such as rifampicin or streptomycin, or an antimycotic such as econazole. Preferred polar lipids include but are not limited to acyl- and acylated carnitine, sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin and phosphatidic acid. Pharmaceutical compositions comprising the drug/polar lipid conjugates of the invention formulated with a medicinal ointment or salve are also provided.

The invention also provides compositions of matter comprising an antiproliferative drug, an antineoplastic drug, an antibiotic, an antimycotic, or an antiviral drug covalently linked to a polar lipid carrier molecule *via* a spacer molecule wherein the spacer allows the drug to act without being released at an intracellular site. In these embodiments of the invention, the first linker functional group attached to the first end of the spacer is characterized as "strong" and the second linker functional group attached to the second end of the spacer is characterized as "weak", with reference to the propensity of the covalent bonds between each end of the spacer molecule to be broken.

In other embodiments of the compositions of matter of the invention, the spacer allows the facilitated hydrolytic release of the antiproliferative, antineoplastic, antibiotic, antimycotic or an antiviral drug at an intracellular site. Other embodiments of the spacer facilitate the enzymatic release of a drug as provided by the invention at an intracellular site. In particularly preferred embodiments, the

spacer functional group is hydrolyzed by an enzymatic activity found in skin, preferably an esterase and most preferably an esterase having a differential expression and activity profile in different skin layers. In additional preferred embodiments, specific release of the antiproliferative, antineoplastic, antibiotic, antimycotic or antiviral drugs of the invention is achieved by enzymatic or chemical release of these drugs by intracellular cleavage of a cleavable linker moiety in cells infected by a pathogenic organism or otherwise expressing a disease state (*for example*, hyperplasia associated with a benign or malignant skin condition) *via* an enzymatic activity specific for such a pathogenic organism or disease state, or by extracellular cleavage of a cleavable linker moiety *via* an enzymatic activity specific for a pathogenic organism or disease state.

In other embodiments of the compositions of matter of the invention, the spacer allows the facilitated enzymatic or hydrolytic release of the antiproliferative, antineoplastic, antibiotic, antimycotic or an antiviral drug at dermal, intradermal and infradermal structures in skin for delivery of therapeutic agents for the treatment of skin diseases and disorders.

In another embodiment of this aspect of the invention, the spacer molecule is a peptide of formula (amino acid)_n, wherein n is an integer between 2 and 25, preferably wherein the peptide comprises a polymer of one or more amino acids.

A preferred embodiments of this aspect of the invention include compositions of matter that are *N*-methotrexate ceramide, methotrexate-glycylglycylglycylglycyl ceramide ester, methotrexate-(tri-β-hydroxypropionylester)-O⁺-ceramide ester, methotrexate-glycylglycylglycylglycyl ceramide ester, methotrexate-aminohexanoyl) sphingosine amide, methotrexate-valinylvalinyl sphingosine amide and methotrexate-O⁺-ceramide ester.

Particular preferred embodiments of the polar lipid/drug conjugates of this invention are provided as salves and other topically or locally applied compositions comprising the drug/polar lipid conjugates of the invention and any of a variety of emollients or other commonly encountered components of cremes, salves, poultices, lotions, gels or other substances well-known in the art for applying compounds to skin and other tissues. Appropriate formulations of such compositions comprising

the drug/ polar lipid conjugates of the invention will be apparent and within the skill of one of ordinary skill in this art to advantageously prepare in view of the instant disclosure.

5 In preferred embodiments, the drug/lipid conjugates of the invention comprise a functionality recognized by an enzymatic activity, most preferably an esterase activity, that has a differential pattern of expression or activity in different skin layers. In additional preferred embodiments, specific release of the antiproliferative, antineoplastic, antibiotic, antimycotic or antiviral drugs of the invention is achieved by enzymatic or chemical release of these drugs by
10 intracellular cleavage of a cleavable linker moiety in cells infected by a pathogenic organism or otherwise expressing a disease state (*for example*, hyperplasia associated with a benign or malignant skin condition) *via* an enzymatic activity specific for such a pathogenic organism or disease state, or by extracellular cleavage of a cleavable linker moiety *via* an enzymatic activity specific for a pathogenic
15 organism or disease state.

As disclosed herein, the invention comprehends a polar lipid-drug conjugate wherein the polar lipid will selectively associate with certain biological membranes, and thereby facilitate entry of the drug into cells and cellular organelles. In
20 embodiments comprising a spacer moiety, the spacer component of the conjugates of the invention will preferably act to release the drug from the lipid, target the conjugate to the cell, or perform other functions to maximize the effectiveness of the drug.

This type of conjugate has numerous advantages. First, the drug-lipid conjugates of the invention promote the intracellular entry of a variety of potentially
25 useful drugs at pharmacokinetic rates not currently attainable. Second, the range of targeted cell types is not limited *per se* by particular, limited biological properties of the cell (such as the number and type of specific receptor molecules expressed on the cell surface). Third, in contrast to traditional attempts to simply target drugs to specific cells, this method may target drugs to specific intracellular organelles
30 and other intracellular compartments. Fourth, the compositions of matter of the invention incorporate a variable spacer region that may allow pharmacologically-

relevant rates of drug release from polar lipid carrier molecules to be engineered into the compositions of the invention, thereby increasing their clinical efficacy and usefulness. Thus, time-dependent drug release and specific drug release in cells expressing the appropriate degradative enzymes are a unique possibility using the drug-lipid conjugates of the invention. Fifth, the conjugates of the invention can be combined with other drug delivery approaches to further increase specificity and to take advantage of useful advances in the art. Sixth, the conjugates of the invention can be topically applied to skin, and the layer of skin penetrated determined by the formulation used. Seventh, in such formulations, the amount and activity of the topically-applied drug can be modulated by release *via* cleavage, preferably hydrolytic cleavage, of the spacer moiety, most preferably by an enzymatic activity in skin that has a differential pattern of expression or activity in different skin layers.

Specific preferred embodiments of the present invention will become evident from the following more detailed description of certain preferred embodiments and the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the synthetic scheme put forth in Example 1.

Figure 2 depicts the synthetic scheme put forth in Example 2.

Figure 3 depicts the synthetic scheme put forth in Example 3.

Figure 4 depicts the synthetic scheme put forth in Example 4.

Figure 5 depicts the synthetic scheme put forth in Example 5.

Figure 6 depicts the synthetic scheme put forth in Example 6.

Figure 7 depicts the synthetic scheme put forth in Example 7.

Figure 8 depicts the synthetic scheme put forth in Example 8.

Figures 9A through 9D depict prodrugs tested as in Example 9.

Figure 10 depicts the synthetic scheme put forth in Example 10.

Figures 11 through 19 illustrate targeting of polar lipid-conjugated biologically active compounds to skin.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention provides compositions of matter and methods for facilitating the entry into cells of biologically-active compounds. For the purposes of this invention, the term "biologically-active compound" is intended to encompass all naturally-occurring or synthetic compounds capable of eliciting a biological response or having an effect, either beneficial or cytotoxic, on biological systems, particularly cells and cellular organelles. These compounds are intended to include but are not limited to all varieties of drugs, particularly antiproliferative drugs and agents, antibacterial, fungicidal, anti-protozoal and antiviral drugs, antineoplastic drugs, and cytotoxic and cytostatic compounds.

Pharmaceutical compositions comprising the drug/polar lipid conjugates of the invention formulated with a medicinal ointment or salve are also provided. As used herein the terms "medicinal ointment or salves" are considered equivalent. The term is intended to encompass any of a variety of salves and other topically or locally applied formulations known in the art, and specifically to encompass any of a variety of emollients or other commonly encountered components of cremes, salves, poultices, lotions, gels or other substances well-known in the art for applying compounds to skin and other tissues. Appropriate formulations of such compositions comprising the drug/ polar lipid conjugates of the invention will be apparent and within the skill of one of ordinary skill in this art to advantageously prepare in view of the instant disclosure.

The compositions of matter provided by the invention comprise the biologically-active compounds of the invention covalently linked to a polar lipid carrier. A polar lipid carrier, as defined herein is intended to mean any polar lipid having an affinity for, or capable of crossing, a biological membrane, including but not limited to sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin, phosphatidic acid, sphingomyelin and other sphingolipids, as these terms are understood in the art (*see, Lehninger, Biochemistry, 2d ed., Chapters 11 & 24, Worth Publishers: New York, 1975*). Additionally, certain other lipids, such as acylated carnitine, comprise the conjugates of the invention (*see Small, 1986,*

"From alkanes to phospholipids," *Handbook of Lipid Research: Physical Chemistry of Lipids*, Volume 4, Chapters 4 and 12, Plenum Press: New York).

The compositions of matter of the invention may be further comprised of a spacer moiety comprising a first end and a second end, each end of the spacer having a functional linking group. For the purposes of this invention, the term "spacer" or "spacer moiety" is intended to encompass any chemical entity that links the biologically-active compound and the polar lipid. Such spacer moieties may be designed to facilitate the attachment of the conjugates of the invention to a target cell, or to facilitate, influence, modulate or regulate the release of the biologically-active compound at the desired target site. Such spacers may also facilitate enzymatic release at certain intracellular sites. Spacer groups, as described herein, include, but are not limited to aminohexanoic acid, polyglycine, polyamides, polyethylenes, and short functionalized polymers having a carbon backbone which is from one to about twelve carbon molecules in length. Particularly preferred embodiments of such spacer moieties comprise peptides of formula (amino acid)_n, wherein n is an integer between 2 and 25 and the peptide is a polymer of one or more amino acids.

The term "linker functional group" is defined herein as any functional group for covalently binding the polar lipid carrier or biologically-active agent to the spacer group. These groups can be designated either "weak" or "strong" based on the stability of the covalent bond which the linker functional group will form between the spacer and either the polar lipid carrier or the biologically-active compound. The weak functionalities include, but are not limited to phosphoramidate, phosphoester, carbonate, amide, carboxyl-phosphoryl anhydride, ester and thioester. The strong functionalities include, but are not limited to ether, thioether, amine, amide and ester. The use of a strong linker functional group between the spacer group and the biologically-active compound will tend to decrease the rate at which the compound will be released at the target site, whereas the use of a weak linker functional group between the spacer group and the compound may act to facilitate release of the compound at the target site. Enzymatic release is, of course, also possible, but such enzyme-mediated modes of release will not necessarily be

correlated with bond strength in such embodiments of the invention. Spacer moieties comprising enzyme active site recognition groups, such as spacer groups comprising peptides having proteolytic cleavage sites therein, are envisioned as being within the scope of the present invention.

5 The drug/polar lipid conjugates of the invention are preferably provided comprised of spacer moieties that impart differential release properties on the conjugates related to differential expression or activity of enzymatic activities in different layers of skin. Biologically active agents such as antiproliferative, antiviral or antineoplastic drugs linked to polar lipids can be delivered to different layers and
10 structures in the skin based on the distinct differences in lipid handling within the skin. Such different structures include dermal, intradermal and infradermal regions or sections of the skin. As shown herein in Example 11, there are important differences in the delivery of a fluorescent marker in mouse skin, based on the polar lipid to which it was linked *via* an amide bond. In addition to distribution by lipid
15 handling, differences in hydrolytic enzymes activity, such as esterases and peptidases, within the skin may be employed to convey specificity to drug distribution.

 For example, hydrolysis of esters within the skin by native esterases has been well documented. Moreover, the unique pattern of metabolites found herein suggested that skin expresses a collection of esterases different than other high esterases
20 containing organs, such as liver (Henrikus and Kampffmeyer, 1992, *Xenobiotica* 22: 1357-1366). Significant variation in substrate specificity among esterases has been shown previously. For example, Heymann *et al.* (1993, *Chem. Biol. Interactions* 87: 217-226) showed the existence of at least three families of β -type esterase, identified by pI and all of which had activity with simple aromatic and aliphatic esters. The rate
25 of ester hydrolysis within skin has been found to be significant. Boehnlein *et al.* (1994, *Pharmaceutical Research* 11: 1155-1159) showed that about half of dermally-applied retinyl palmitate was hydrolyzed within a few minutes; similar results were reported with methyl salicylate. β -methasone-17-valerate, a steroid fatty acid ester, has also been reported to be hydrolyzed at a pharmacologically-important rate by skin
30 esterases (Kubota *et al.*, 1994, *Dermatology* 188: 13-17). In addition, smaller molecules are delivered more efficiently to skin linkage with lipids. For example,

Buyuktimkin *et al.* (1993, *Pharmaceutical Research* 10:1632-1637) found that uptake of indomethacin and clonidine could be enhanced by linkage to a polar lipid.

Distribution of specific esterases within the skin has not been extensively studied. It is known that some cell types do possess different constituent esterases. The Langerhans cells contain an α -naphthylacetate hydrolyzine esterase, used in antigen recognition and processing, that is not found in other cell types (Lipozencic *et al.*, 1994, *Eur. J. Histochem.* 38: 303-310). Keratinocytes are also known to have high levels of chloroacetate esterase (Katz *et al.*, 1995, *Brit. J. Dermatology* 133: 842-846).

Esterase activity is also known to vary with pathological conditions. Nonspecific esterase activity increases in cells around the edge of many types of wounds (Dachun *et al.*, 1992, *Forensic Science International* 53: 203-213). Although not produced by skin cells *per se*, esterases are known to increase in the skin due to influx of other cell types during illness. One example is chloroacetate esterase activity is increased in any condition causing chronic urticaria due to mast cell infiltration (Barlow *et al.*, 1995, *Clinical & Experimental Allergy* 25: 317-322). In another example, monocytes and macrophages found in the skin in leprosy increase esterase activity (SivaSai *et al.*, 1993, *Int. J. Leprosy & Other Mycobacterial Diseases* 61: 259-269).

Other enzymatic activities are associated with skin. Peptidases are found in high levels within skin tissues and the non-dermal cell types infiltrating the skin. Expression of both endo- and exo-peptidases and proteases varies with cell type and pathological condition. Skin fibroblasts contain high levels of peptidases, not seen in other more highly differentiated layers of skin (Bou-Gharios *et al.*, 1995, *Annals of Rheumatic Disease* 54: 111-116). High levels of dipeptidyl dipeptidase IV are seen in precancerous dermatoses and basal cell carcinoma (Moehrle *et al.*, 1995, *J. Cutaneous Pathology* 22: 241-247). Increased peptidase activity is associated with stress responses: exemplary are apoptotic skin cells produced after UV irradiation (so-called sunburn cells), which have high amino and endopeptidase activity: Brown *et al.* (1994, *J. Cellular Biochemistry* 54: 320-331) have suggested that these apoptotic keratinocytes may be essential elements in the repair process for epithelial cells.

The current state of knowledge of these hydrolytic enzymes in skin is not extensive relative to other organs. However, sufficient data is available to see that there are differences in substrate specificity and enzyme activity that can be exploited to further refine the specificity of the polar lipid delivery vehicle. Formulation of cleavable linkages directed at these enzymes, combined with the larger distributive properties of the lipid carries could provide important new ways to deliver pharmaceutical agents to highly defined locations in the skin, dependent on either normal skin or pathological conditions. Conjugates that advantageously utilize the differential expression and/or activity levels of esterases, proteases, and other enzymatic functionalities are provided by this invention.

As provided by this invention, the specificity of the cleavage of the linker moiety as provided by this invention is the result of the combination of particular linker moieties selected to be specifically cleaved inside an infected skin cell. In one aspect, such specific cleavage is due to an chemical linkage which is labile within the infected cell due to conditions caused by or that result from infection of the cell with a particular pathogenic organism. In another aspect, such specific cleavage is due to an enzymatic activity which is produced either by the pathogen itself or by the cell as the result of infection with said pathogen, wherein the linkage is enzymatically cleaved by the enzymatic activity. Similarly, specific cleavage is obtained in cells expressing a disease or pathologic state, whereby a chemical linkage labile to the conditions inside the diseased cell or enzymatic cleavage due to an enzymatic activity expressed in a diseased cell using drug/polar lipid conjugates comprising the appropriate linking moiety comprising the labile chemical linkage or enzyme recognition site. Differences in skin and other tissues present extracellularly due to the expression of a disease state or the presence of a pathogenic organism are also comprehended to be within the scope of the invention.

Examples of such combinations resulting in specific release of an antimicrobial drug embodiment of the invention within infected cells include but are not limited to a urea-based linker for use against a pathogen which produces urease (e.g., *Mycobacteria spp.* and *B. pertussis*); a peptide linker comprised of (AlaAlaAlaAla)_n, wherein n can be an integer from 1-5, for use against a pathogen

that produces the protease oligopeptidase A (*e.g.*, *Salmonella spp.*); a peptide comprised of from 3 to about 20 amino acids comprising the sequence --Pro-Xaa-Pro--, where Xaa is any amino acid, for use against a pathogen that produced proline peptidase (*e.g.*, *Salmonella spp.*); peptides comprising the dipeptide MetMet or LeuAla, or peptides comprising the amino acid sequence GSHLVEAL, HLVRALYL, VEALYLVC, or EALYLVCG, for use against human immunodeficiency virus 1 producing a specific protease termed HIV-1 protease; a peptide comprising the amino acid sequence: -Ala-Xaa-Cys_{Ac_m}-Tyr-Cys-Arg-Ile-Pro-Ala-Cys_{Ac_m}-Ile-Ala-Gly-Asp-Arg-Arg-Tyr-Gly-Thr-Cys_{Ac_m}-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys_{Ac_m}-Cys_{Ac_m}-, wherein the pathogen expresses an enzymatic activity that specifically disables the endogenous antimicrobial peptide defensin (*e.g.*, *Mycobacterium spp.* and *L. pneumophila*), (-Cys_{Ac_m}-) represent cysteine residues having the sidechain sulfur atom protected by covalent linkage to an acetamidomethyl group (it will be recognized that embodiments of such peptides having alternative sulfur protecting groups are also within the scope of the disclosure herein) and Xaa is either absent or Asp (said peptides are also useful against a pathogen such as *Legionella spp.* producing a 39 kDa metalloprotease); hippurate esters that are hydrolyzed by pathogen-specific (*e.g.*, *L. pneumophila* and *Listeria spp.*) hydrolase; nicotinic acid amides cleaved by nicotinamidases, pyrazinamides cleaved by pyrazinamidase; allolactose linkages cleaved by β -galactosidase; and allantoate linkages cleaved by allantoicase (*e.g.*, *Mycobacterium spp.*).

Thus, the present invention provides methods and compositions of matter for facilitating the entry of antiproliferative, antibiotic, antimycotic, antiviral and antineoplastic agents, drugs and compounds into dermal and epidermal cells, across mucosal membranes where appropriate, and distributed within skin tissue for efficient delivery of such compounds locally and topically for the treatment of animal, preferably human, diseases and pathological conditions. The invention provides salves, ointments, poultices and other topically-applied embodiments of the drug/lipid conjugates of the invention for the treatment of a variety of skin diseases and disorders.

Among the most common dermatological complaints is dermatitis, further differentiated into contact, seborrheic, nummular, exfoliative stasis, and neurodermatitis. Vesicular dermatitis, commonly referred to as eczema, is separated from other dermatitis to reflect the chronic nature of the condition. All forms of dermatitis are characterized by superficial inflammation, vesiculation and localized edema. Accompanying these psoriasiform conditions is thickening of the epidermis showing both hyperkeratosis and parakeratosis. Topical corticosteroids are widely prescribed for these conditions, including the Potency class I drugs betamethasone dipropionate, clobetasol propionate, diflorasone diacetate and flucinolone. Of particular interest in the treatment of psoriasis is methotrexate, the use of which is severely limited by the nephro- and hepatotoxicity. In a preferred embodiment, the invention provides a conjugate, methotrexate ceramide ester (ME6C), that does not concentrate in either liver or kidney to the same extent as the free drug, and is therefore useful in for treatment of chronic psoriasis and related conditions.

Another skin condition conventionally treated by topical application of an antineoplastic agent is the treatment of precancerous lesions classified as actinic keratoses. These lesions respond to 5-fluorouracil (5-FU) topically applied in a propylene glycol carrier. However, topical application of 5-FU is limited by the toxicity of this compound in the systemic circulation. Topical application can be improved by covalent, hydrolyzable linkage of 5-FU to polar lipids that is cleaved by an esterase or peptidase activity in skin. Such compounds have the advantage of better penetration, longer retention and controlled release of active drug.

Superficial fungal infections by the genera *Microsporum*, *Trichophyton*, and *Epidermophyton*, the so-called dermatophyte infections, are extremely common. Ringworm and the *Tenia* infections, including candidiasis, colonize the area between the dead skin and living layers eliciting vesicular and bullous diseases as well as inflamed lesions of the scalp due to strong immunological responses to the fungi. All respond, to some extent, to topical antifungals. Topical application reduces reliance on systemic antifungals, avoiding the hepatic toxicity produced by administration of the currently used antifungals such as ketoconazole, griseofulvin, ciclopirox, naftifine and other imidazole antimycotics. By conjugating these

compounds with polar lipid carriers, these drugs are delivered to specific areas of the skin in greater quantity and maintained for longer periods of time as a consequence of the lipid formulation. Release of active drug from these reservoirs is effectuated by the cleavage of hydrolyzable bonds between the drug and the lipid carrier. By relying on the specific partitioning effect of the polar lipids, a greater therapeutic index is thereby achieved, thus reducing the need for system antimycotics and reducing the risk of hepatic toxicity. In addition, the characteristic accumulation of fluids in eczema, or other bullus conditions, may be a limiting factor in the delivery of water soluble antimycotics. Use of the lipid prodrugs to effectively deliver antifungals to deeper skin structures would improve treatment of eczema and yeast-exacerbated dermatitis.

In skin infestations and dermatitis, deep colonization or involvement of sub-epidermal structures is common. Delivery of drugs to deep skin structures is important for relief of these conditions. The conjugation of compounds to polar lipids, as shown in Example 10 below, indicates that transport and release of medicaments through the skin is possible.

The invention provides polar lipid/drug conjugates comprising corticosteroids, including but not limited to cortisone, cortisol, hydrocortisone, prednisone, fluorinated corticosteroids (such as fluocinalone and triamcinolone), dexamethasone, alcloethasone, fluoroandrenolide and mometasone.

The invention provides polar lipid/drug conjugates comprising antimycotic compounds including but not limited to clotrimazole, ciclopirox, nystatin, econazole and myconixole. The invention provides polar lipid/drug conjugates comprising antibiotics including but not limited to penicillin and drugs of the penicillin family of antimicrobial drugs, including but not limited to penicillin-G, penicillin-V, phenethicillin, ampicillin, amoxicillin, cyclacillin, bacampicillin, hetacillin, cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin, azlocillin, carbenicillin, mezlocillin, piperacillin, ticarcillin, and imipenim; cephalosporin and drugs of the cephalosporin family, including but not limited to cefadroxil, cefazolin, cephalexin, cephalothin, cephalixin, cephradine, cefaclor, cefamandole, cefonicid, cefoxin, cefuroxime, ceforanide, cefotetan, cefmetazole, cefoperazone, cefotaxime,

ceftizoxime, ceftizone, moxalactam, ceftazidime, and cefixime; aminoglycoside drugs and drugs of the aminoglycoside family, including but not limited to streptomycin, neomycin, kanamycin, gentamycin, tobramycin, amikacin, and netilmicin; macrolide and drugs of the macrolide family, exemplified by
5 azithromycin, clarithromycin, roxithromycin, erythromycin, lincomycin, and clindamycin; tetracyclin and drugs of the tetracyclin family, for example, tetracyclin, oxytetracyclin, democlocyclin, methacyclin, doxycyclin, and minocyclin; quinoline and quinoline-like drugs, such as, for example, naladixic acid, cinoxacin, norfloxacin, ciprofloxacin, ofloxacin, enoxacin, and pefloxacin; antimicrobial
10 peptides, including but not limited to polymixin B, colistin, and bacatracin, as well as other antimicrobial peptides such as defensins (Lehrer *et al.*, 1991, *Cell* 64: 229-230), magainins (Zasloff, 1987, *Proc. Natl. Acad. Sci. USA* 84: 5449-5453), cecropins (Lee *et al.*, 1989, *Proc. Natl. Acad. Sci. USA* 86: 9159-9162 and Boman *et al.*, 1990, *Eur. J. Biochem.* 201: 23-31), and others, provided as naturally-
15 occurring or as the result of engineering to make such peptides resistant to the action of deactivating enzymes; other antibiotic drugs, including chloramphenicol, vancomycin, rifampicin, metronidazole, ethambutol, pyrazinamide, sulfonamides, isoniazid, and erythromycin.

The invention also provides polar lipid/drug conjugates of antiviral agents,
20 including but not limited to reverse transcriptase inhibitors, protease inhibitors, antiherpetics such as acyclovir and gangcyclovir, azidothymidine, cytidine arabinoside, ribavirin, amantadine, iododeoxyuridine, poscarnet, trifluoridine, methizazone, vidarabine and levanisole.

The invention provides polar lipid/drug conjugates of antiproliferative and
25 antineoplastic agents, including but not limited to methotrexate, doxorubicin, daunorubicin, actinomycin D, vinblastine, vincristine, colchicine and taxol.

The invention specifically provides methods for preparing and administering such antiproliferative compounds for use in treating pathological conditions *in vivo*.

Animals to be treated with polar lipid-antiproliferative agent conjugates using
30 the methods of the invention are intended to include all vertebrate animals,

preferably domesticated animals, such as cattle, horses, goats, sheep, fowl, fish, household pets, and others, as well as wild animals, and most preferably humans.

The following Examples illustrate certain aspects of the above-described method and advantageous results. The following examples are shown by way of illustration and not by way of limitation.

EXAMPLE 1

An antibiotic drug/polar lipid conjugate of the invention is prepared by conjugating a specifically-cleavable peptide to a polar lipid and an antibiotic drug as follows. An derivatized polar lipid comprising unconjugated amino groups is reacted with a proteolytically-inert peptide in which the terminal amine and any of the constituent amino acid sidechain reactive amines are covered by *tert*-butoxycarbonyl (*t*-Boc) protecting groups in the presence of triphenyl phosphine as described by Kishimoto (1975, *Chem. Phys. Lipids* **15**: 33-36). The peptide/polar lipid conjugate is then reacted in the presence of pyridine hydrofluoride as described by Matsuura *et al.* (1976, *J. Chem. Soc. Chem. Comm.* **xx**: 451-459) to remove the *t*-Boc protecting groups. The peptide/polar lipid is then conjugated to the specifically-cleavable peptide, in which the terminal amine and any of the constituent amino acid sidechain reactive amines are covered by *t*-Boc protecting groups, as described in the presence of triphenyl phosphine. After deprotection of reactive amines with pyridine hydrofluoride as described, an antibiotic drug having a reactive carboxylic acid group is conjugated to a free amino group of the polar lipid/peptide/specifically-cleavable peptide to yield the antibiotic drug/polar lipid conjugate of the invention. This reaction scheme is illustrated in Figure 1.

EXAMPLE 2

An antiviral compound (HIV1 protease inhibitor; compound 8) is conjugated to sphingosine as follows. Sphingosine is reacted with 1,3 *bis*-(trimethylsilyl)urea as described by Verbloom *et al.* (1981, *Synthesis* **1032**: 807-809) to give a trimethylsilyl derivative of sphingosine. The sphingosine derivative is then

conjugated with a specifically-cleavable peptide in which the terminal amine and any of the constituent amino acid sidechain reactive amines are covered by *tert*-butoxycarbonyl (*t*-Boc) protecting groups in the presence of diethylazo-dicarboxylate (DEAD) and triphenyl phosphine as described by Kishimoto (1975, *Chem. Phys. Lipids* 15: 33-36). The sphingosine/peptide conjugate is then reacted in the presence of pyridine hydrofluoride as described by Matsuura *et al.* (1976, *J. Chem. Soc. Chem. Comm.* xx: 451-459) to remove the *t*-Boc protecting group, to yield the peptide covalently linked to sphingosine through an amide bond. This reaction scheme is illustrated in Figure 2. Sphingosine/peptide conjugates are then linked to the antiviral compound as described in Example 1.

EXAMPLE 3

An antiviral compound (compound 8) is conjugated to ceramide via a polyglycine spacer as follows and as illustrated in Figure 3. The amino terminus of polyglycine is protected by a *t*-Boc group. Polyglycine is conjugated through its carboxy terminus to ceramide forming an ester linkage, as described in Anderson *et al.*, *ibid.* The resulting compound is then conjugated through the amino terminus of the polyglycine residue. The amino terminus of Compound 8 is also protected by a *t*-Boc protecting group. Conjugation with polyglycyl-sphingosine takes place between the amino terminus of the polyglycyl spacer moiety and the carboxy terminus of the HIV-1 protease inhibitor. This reaction is carried out in the presence of DEAD and triphenyl phosphine as described in Examples 1 and 2. Following this conjugation, the amino terminus of the HIV-1 protease inhibitor residue is deprotected according to the method of Matsuura *et al.*, *ibid.*

EXAMPLE 4

An antiviral compound is prepared wherein ceramide is first conjugated to a first end of an oligomeric 3-hydroxy propanoic acid spacer through an ester functional group, and wherein AZT is conjugated to a second end of said polyester spacer through a phosphodiester bond. First a polyester spacer is obtained, having a carboxyl at a first end and a triphenylmethyl group esterified to a second end.

This spacer is conjugated to ceramide at its first end through an ester functional linker group according to the method of Anderson *et al.*, *ibid.* This compound is then conjugated through the second end of the spacer compound to AZT monophosphate by means of a phosphodiester bond according to the method of Baer
5 (1955, *Can. J. Biochem. Phys.* 34: 288). In this antiviral compound, the bond breakage between the spacer and the drug would be slow in the absence of a phosphohydrolase. This reaction scheme is illustrated in Figure 4.

EXAMPLE 5

10 An antiviral compound wherein phosphatidic acid, phosphatidyl choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol or phosphatidylethanolamine is linked through a phosphoester linker functional group to the antiviral drug azidothymidine (AZT). Phosphatidic acid, phosphatidyl
15 choline, phosphatidyl serine, phosphatidyl inositol, phosphatidyl glycerol or phosphatidyl ethanolamine is conjugated to AZT according to the method of Salord *et al.* (1986, *Biochim. Biophys. Acta* 886: 64-75). This reaction scheme is illustrated in Figure 5.

EXAMPLE 6

20 An antiviral compound is prepared wherein aminohexanoyl sphingosine is conjugated to AZT. Aminohexanoyl sphingosine is conjugated with AZT according to the method of Kishimoto (1975, *Chem. Phys. Lipid* 15: 33-36). This reaction scheme is illustrated in Figure 6 to yield aminohexanoyl sphingosine conjugated to
25 AZT through a phosphoramidate bond.

EXAMPLE 7

An antiviral compound consisting of ceramide conjugated to AZT-monophosphate is provided. Ceramide is reacted with AZT-monophosphate in the presence of dicyclohexylcarbodiimide as described in Smith and Khorana (1958, *J. Amer. Chem. Soc.* 80: 1141) to yield ceramide conjugated through a phosphodiester
30 bond to AZT-monophosphate. This reaction scheme is illustrated in Figure 7.

EXAMPLE 8

An antiviral compound is prepared wherein ceramide is conjugated through an ester functional group to a first end of a polyglycine spacer, and wherein AZT is conjugated through a phosphoester functional group to a second end of the polyglycine spacer. Ceramide is first conjugated through an ester functional group to a first end of a polyglycine spacer (as described in Example 2). The ceramide-polyglycine compound is then conjugated through a phosphoester bond to a second end of the polyglycine spacer to AZT monophosphate according to the method of Paul and Anderson, *ibid*. This reaction scheme is illustrated in Figure 8.

EXAMPLE 9

The effect of presenting a biologically active compound such as a drug to mammalian cells as a prodrug covalently linked to a polar lipid carrier moiety was determined as follows. The antifolate drug methotrexate was conjugated with a variety of polar lipid carriers *via* organic spacer moieties having specific reactive functional groups. A representative sample of such compounds is shown in Figures 9A through 9C, wherein MC represents Mtx linked to sphingosine *via* an amide bond to a 6-aminohexanoic acid spacer, ME₆C represents Mtx linked to sphingosine *via* an ester linkage to a 6-hydroxyhexanoic acid spacer, and MSC represents Mtx linked to sphingosine *via* a salicylic acid ester linkage to a 6-aminohexanoic acid spacer. Also studied was a conjugate of azidothymidine linked to sphingosine *via* an ester linkage to a 6-hydroxyhexanoic acid spacer (N-AZT-ceramide; Figure 9D). The compounds were tested for their growth inhibitory effects on murine NIH 3T3 cells growing in cell culture. About one million such cells per P100 tissue culture plate were grown in DMEM media supplemented with 10% fetal calf serum (GIBCO, Grand island, NY) in the presence or absence of a growth-inhibitory equivalent of each prodrug. Cell numbers were determined after 70 hours growth in the presence or absence of the prodrug. In a second set of experiments was included in the growth media an amount of a brain homogenate containing an enzymatically-active esterase.

The results from these experiments are shown in Table I. As can be seen from these data, the MC prodrug had no effect on the growth and survival of the cells. This result did not change upon co-incubation with the esterase-containing brain extract, which was expected due to the nature of the drug/spacer linkage (an amide bond). A different result was obtained with the ME₆C conjugate. The prodrug was ineffective in inhibiting cell growth or survival in the absence of brain extract. Upon addition of the brain extract, a significant increase in Mtx cytotoxicity was observed. This is consistent with cleavage of the ester linkage by the brain extract-derived esterase. A similar result was obtained with the MCS conjugate, indicating that the brain extract esterase activity was capable of cleaving the salicylic acid ester.

Table II shows the results of drug uptake studies performed with the prodrug N-AZT-ceramide. Antiviral amounts of the prodrug conjugate were added to NIH 3T3 cell cultures, and the antiviral activity of the prodrug was found to be equivalent to the activity of free AZT. In addition, upon removal of the prodrug, intracellular retention of prodrug was found to be up to 15-fold higher than free AZT (Table II) over a 23h period.

These results indicate that for Mtx-containing conjugates, the free drug must be released from the prodrug for biological activity. These results suggest that specific release of this drug, and perhaps others, can be achieved using cleavable linker moieties that are specifically cleaved only in pathogen-infected cells.

TABLE I

<u>Sample¹</u>	<u># cells/plate²</u>	<u>Sample³</u>	<u># cells/plate⁴</u>
Control/FBS	7.8 x 10 ⁶	Control/FBS	13 x 10 ⁶
ME ₆ C/FBS	6.5 x 10 ⁶	MSC/FBS	2.1 x 10 ⁶
ME ₆ C/brain	2.7 x 10 ⁶	MSC/brain	0.51 x 10 ⁶
Mtx/FBS	0.16 x 10 ⁶	Mtx/FBS	0.13 x 10 ⁶
Mtx/brain	0.09 x 10 ⁶	Mtx/brain	0.06 x 10 ⁶
Control/brain	N.D.	Control/brain	6.2 x 10 ⁶

¹ = cells incubated with drug/FBS or drug/brain extract for 1 hour at 37°C

² = cell growth and survival determined 70 hours after drug addition

³ = cells incubated with drug/FBS or drug/brain extract for 2 hours at 37°C

⁴ = cell growth and survival determined 72 hours after drug addition

TABLE II

<u>Time¹</u>	<u>AZT²</u>	<u>N-AZT-Ceramide²</u>
0 hr.	6.49	8.45
23 hr.	0.55	7.78

¹ = time between the end of drug treatment and assay for intracellular drug concentration

² = nM/10⁶ cells

EXAMPLE 10

An antiproliferative agent is prepared wherein the anti-proliferative drug methotrexate (Mtx) is conjugated to sphingosine *via* a 6-aminocaproic acid spacer.

This reaction scheme is illustrated in Figure 10. The primary amino and hydroxyl groups of sphingosine are acylated by reaction with activated *N*-(methotrexate)aminocaproic acid overnight at 40-50°C, followed by base hydrolysis

in 0.1N methanolic KOH. The Mtx derivative of 6-aminocaproic acid is synthesized by activating the carboxylic acid moiety of Mtx and reacting with 6-aminocaproic acid for 2 days at 60-70°C. This reaction is stopped under acidic conditions to liberate anhydrides that form under these conditions.

5

EXAMPLE 11

An *in vivo* mouse skin model system was used to demonstrate the use of embodiments of the polar lipid conjugates of the invention for introducing biologically-active compounds through the epidermal layer of the skin and into the underlying skin layers.

10

In these experiments, various embodiments of the polar lipids of the invention were conjugated to a fluorescent compound, (7-nitro-2-1,3-benzoxadiazol-4-yl)-hexanoate (NBD), conjugation being achieved using the methods disclosed herein (Example 10). The NBD-polar lipid conjugates were mixed with dimethylsulfoxide (DMSO), and 20 μ L of a 1.7% solution of each conjugate in DMSO were applied to shaved mouse skin and allowed to penetrate the skin for 4 hours. After the 4 hour incubation, skin sections were excised and prepared for light or fluorescence microscopy, using standard histological techniques.

15

The results of these experiments are shown in Figures 11 through 19. In each Figure, the outer layer of the epidermis is located in the upper, left-hand corner of the photomicrograph.

20

Figure 11 is a photomicrograph that illustrates hematoxylin-eosin (H&E) staining of mouse skin, observed by light microscopy under 100X magnification. It was observed in this photomicrograph that H&E staining was concentrated in the epidermis and reticular dermis, and that the papillary dermis remained relatively unstained.

25

In comparison, Figure 12 is a fluorescence photomicrograph that illustrates ceramide-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification. In this Figure it was observed that the ceramide-NBD fluorescence was carried through the stratum granulosum and epidermis. No partitioning into keratinocytes or Langerhans cells was observed, but distribution

30

through the skin section appeared to be cell-dependent, that is, fluorescence was evenly distributed throughout the cells in the section, rather than being distributed nonspecifically through the microscopic field of view.

Figure 13 is a fluorescence photomicrograph that illustrates phosphatidylcholine-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification. Figure 14 is a fluorescence photomicrograph that illustrates phosphatidylethanolamine-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification, and Figure 15 is a fluorescence photomicrograph that illustrates phosphatidylserine-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification. Each of these conjugates was observed to result in localized fluorescence in the outer layers of the skin. In Figures 13 and 15, some staining of specific areas below the epidermis was also observed, and the compound of Figure 15 was observed to penetrate into the papillary dermis.

Figure 16 is a fluorescence photomicrograph that illustrates 1-R{6[(7-nitro-2-1,3-benzoxadiazol-4-ethylamino)caproyl]}-NBD (termed phospho-rac-(1-glycerol)-NBD(caproyl)) staining of mouse skin, observed by fluorescence microscopy under 100X magnification. It was observed that the phospho-rac-(1-glycerol)-NBD(caproyl) conjugate penetrated the skin extensively, but in a pattern distinct and different from ceramide-NBD shown in Figure 12. It was observed that the ceramide-NBD distributed through the cell structure of the deeper skin layers, while the phospho-rac-(1-glycerol)-NBD(caproyl) conjugate concentrated mainly in plasma membranes of fat cells, as well as in some unidentified structures.

Figure 17 is a fluorescence photomicrograph that illustrates phosphatidylserine-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification. The compound used in Figure 17 differs from the phosphatidylserine-NBD conjugate shown in Figure 15 in that the NBD dye is conjugated to the polar lipid *via* a dodecanoyl moiety in the compound of Figure 17 and *via* a caproyl moiety in the compound of Figure 15. In contrast to the results obtained with the compound of Figure 15, the dodecanoyl-conjugated

NBD compound of Figure 17 penetrated into the cornified layer, with only minimal penetration into the epidermis.

Figure 18 is a fluorescence photomicrograph that illustrates 1-R(12{(7-nitro-2-1,3-benzoxadiazol-4-ethylamino))dodecanoyl)-NBD (termed phospho-rac-(1-glycerol)-NBD(dodecanoyl)) staining of mouse skin, observed by fluorescence microscopy under 100X magnification. This compound is related to the compound of Figure 16, but differs in the size of the acyl chain to which the fluorescent NBD label is conjugated; here, it is a dodecanoyl chain, while in Figure 16 it is a caproyl chain. Both the compound of Figure 16 and the instant compound were observed to penetrate the papillary dermis and the reticular dermis. In addition, the dodecanoyl-containing compound of Figure 18 was also observed to accumulate in hair follicles.

Figure 19 is a fluorescence photomicrograph that illustrates phosphatidylethanolamine-NBD staining of mouse skin, observed by fluorescence microscopy under 100X magnification. The compound used in Figure 19 differs from the phosphatidylethanolamine-NBD conjugate shown in Figure 14 in that the NBD dye is conjugated to the polar lipid *via* a dodecanoyl moiety in the compound of Figure 19 and *via* a caproyl moiety in the compound of Figure 14. In contrast to the results obtained with the compound of Figure 14, the dodecanoyl-conjugated NBD compound of Figure 19 penetrated into the dermis.

Since all compounds were administered in the DMSO vehicle, lack of penetration of some compounds into some or most skin layers discounts the possibility that the DMSO vehicle was responsible for non-specifically carrying fluorophore into the tissue.

These results demonstrate that certain of these conjugates showed specific partitioning into defined layers of the skin. Ceramide-NBD, phospho-rac-(1-glycerol)-NBD(caproyl) and phospho-rac-(1-glycerol)-NBD(dodecanoyl) penetrated the skin to the reticular dermis. Caproyl-conjugated phosphatidylethanolamine-NBD, in contrast, did not penetrate beyond the outermost layers of the epidermis, while dodecanoyl-conjugated phosphatidylethanolamine-NBD was observed to penetrate into the dermis. On the other hand, caproyl-conjugated

phosphatidylserine-NBD penetrated into the papillary dermis, while dodecanoyl-conjugated phosphatidylethanolamine-NBD did not penetrate past the cornified layer of the epidermis. These results suggested that polar lipid composition is a determinant in the penetrating ability of the conjugates of the invention, and demonstrated that linkage to polar lipids produced increased penetration of the skin by non-penetrating compounds. These results also demonstrated that the conjugates of the invention partitioned selectively in skin layers and cells, depending on the lipid carrier used in the conjugate. These results further indicate that conjugating antiproliferative compounds of the invention with polar lipids can be used to deliver drugs to specific areas of the skin in greater quantity and concentration than can currently be achieved, and that such drugs can be maintained in specific areas and cells in the skin for longer periods of time. As a consequence of lipid-drug formulation, release of active drug from these conjugates can be achieved by the use of hydrolyzable bonds between drug and carrier.

The specific partitioning of the conjugates of the invention, achieved through the use of polar lipid conjugates, also permits a greater therapeutic index to be achieved. These capacities of the antiproliferative drug conjugates of the invention have important applications to the delivery of medicinal compounds into the skin to treat a variety of pathological conditions. Medicinal salves and ointments for topical treatment purposes are known in the prior art for the treatment of a variety of pathological conditions, but they suffer from non-specific deposition of the antiproliferative drug into both healthy and affected portions of the skin. In addition, appropriate concentrations of topically-applied antiproliferative drugs are currently limited by the escape of the active agent(s) into the systemic circulation, with deleterious effects on other tissues and organs. An example of such a situation is the use of the drug methotrexate to treat psoriasis, where the amount of methotrexate that is capable of being topically applied is limited by hepato- and nephrotoxicity caused by systemic escape of the compound from the skin.

One advantage of the methotrexate-containing embodiments of conjugates of the invention (such as methotrexate ceramide ester, ME₆C), is that this compound

does not concentrate in the liver or kidney to the same extent as free drug, even upon escape into the systemic circulation.

5 Similarly, treatment of fungal infections in the skin is limited by systemic hepatotoxicity of many topically-applied antifungal agents, such as ketoconazole, griseofulvin, and ciclopirox. Specific localization of such compounds to the skin using polar lipid-drug conjugates of the invention provides a means of increasing the dosages of such antifungal agents that can be topically applied. Other uses of the conjugates of the invention include treatment of precancerous lesions with polar lipid conjugated 5-fluorouracil.

10 The present invention therefore solves a problem common to treatment of a variety of pathological conditions in skin tissue with topically-applied salves, ointments or similar medicaments.

15 It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

What is claimed is:

1. A pharmaceutical composition comprising an antiproliferative, antibiotic, antimycotic, antiviral or antineoplastic drug, a polar lipid carrier, two linker functional groups and a spacer, wherein the spacer has a first end and a second end and wherein the polar lipid is attached to the first end of the spacer through a first linker functional group and the drug is attached to the second end of the spacer through a second linker functional group, the composition further comprising a medicinal ointment or salve.
2. The pharmaceutical composition of Claim 1 wherein the drug is methotrexate.
3. A pharmaceutical composition according to Claim 1 wherein the spacer allows the drug to act without being released at an intracellular site and wherein the first linker functional group attached to the first end of the spacer is strong and the second linker functional group attached to the second end of the spacer is weak.
4. A pharmaceutical composition according to Claim 1 wherein the spacer allows the facilitated hydrolytic release of the drug at an intracellular site and wherein the first linker functional group attached to the first end of the spacer is strong and the second linker functional group attached to the second end of the spacer is weak.
5. A pharmaceutical composition according to Claim 1 wherein the spacer allows the facilitated enzymatic release of the drug at an intracellular site and wherein the first linker functional group attached to the first end of the spacer is strong and the second linker functional group attached to the second end of the spacer is weak.
6. A pharmaceutical composition according to Claim 1 wherein the polar lipid is acyl carnitine, acylated carnitine, sphingosine, ceramide, phosphatidyl choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin or phosphatidic acid.
7. A pharmaceutical composition comprising an antiproliferative, antibiotic, antimycotic, antiviral or antineoplastic drug having a first functional

linker group, and a polar lipid carrier having a second functional linker group, wherein the drug is covalently linked to the polar lipid carrier by a chemical bond between the first and second functional linker groups, the composition further comprising a medicinal ointment or salve.

5 8. A pharmaceutical composition according to Claim 7 wherein the first functional linker group is a hydroxyl group, a primary or secondary amino group, a phosphate group or substituted derivatives thereof or a carboxylic acid group.

 9. A pharmaceutical composition according to Claim 7 wherein the second functional linker group is a hydroxyl group, a primary or secondary amino
10 group, a phosphate group or substituted derivatives thereof or a carboxylic acid group.

 10. A pharmaceutical composition according to Claim 7 wherein the polar lipid is acyl carnitine, acylated carnitine, sphingosine, ceramide, phosphatidyl
15 choline, phosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, phosphatidyl serine, cardiolipin or phosphatidic acid.

 11. The pharmaceutical composition of Claim 7 wherein the drug is methotrexate.

 12. Use of a pharmaceutical composition according to Claim 1 for preparing a medicament for treating a pathological condition or disease state in
20 cells, tissues or organs in an animal, in an acceptable carrier or formulation and in an amount sufficient to alleviate the pathological condition or disease state in the animal.

 13. Use of a pharmaceutical composition according to Claim 1 for treating a pathological condition or disease state in skin of an animal, wherein the
25 pathological condition or disease state results from an abnormal proliferation of cells in the animal, in an acceptable carrier or formulation and in an amount sufficient to alleviate the pathological condition or disease state in the animal.

 14. A use according to Claims 12 or 13 wherein the animal is a human.

 15. Use of a pharmaceutical composition according to Claim 7 for
30 preparing a medicament for treating a pathological condition or disease state in cells, tissues or organs in an animal, in an acceptable carrier or formulation and in

an amount sufficient to alleviate the pathological condition or disease state in the animal.

16. Use of a pharmaceutical composition according to Claim 7 for treating a pathological condition or disease state in skin of an animal, wherein the pathological condition or disease state results from an abnormal proliferation of cells in the animal, in an acceptable carrier or formulation and in an amount sufficient to alleviate the pathological condition or disease state in the animal.

17. A use according to Claims 15 or 16 wherein the animal is a human.

18. A pharmaceutical composition according to Claims 1 or 7 wherein the spacer is a peptide of formula (amino acid)_n, wherein n is an integer between 2 and 25, and the peptide comprises a polymer of one or more amino acids.

19. A pharmaceutical composition according to Claim 1 comprising methotrexate glycylglycylglycylglycyl ceramide ester.

20. A pharmaceutical composition according to Claim 1 comprising methotrexate-{tri-β-hydroxypropionylester}-O⁺-ceramide ester.

21. A pharmaceutical composition according to Claim 1 comprising methotrexate (aminohexanoyl)sphingosine amide.

22. A pharmaceutical composition according to Claim 1 comprising methotrexate valinylvalinyl sphingosine amide.

23. A pharmaceutical composition according to Claim 7 comprising methotrexate-O⁺-ceramide ester.

24. A pharmaceutical composition according to Claim 7 comprising N-methotrexate ceramide.

25. A use according to Claims 12, 13, 14, 15, 16 or 17, wherein the disease state is a skin disease.

26. A pharmaceutical composition according to Claim 1 wherein the spacer is a cleavable linker moiety that is specifically cleaved inside a mammalian cell infected with a microorganism or expressing a disease state.

27. The pharmaceutical composition of Claim 26 wherein the cleavable linker moiety is chemically cleaved inside a mammalian cell infected with a microorganism or expressing a disease state.

28. The pharmaceutical composition of Claim 26 wherein the cleavable linker moiety is a substrate for a protein having an enzymatic activity, said protein being specifically expressed in a mammalian cell infected with a microorganism or expressing a disease state.

5 29. A pharmaceutical composition according to Claim 7 wherein the chemical bond linking the polar lipid and the antiproliferative, antibiotic, antimycotic, antiviral or antineoplastic drug is specifically cleaved inside a mammalian cell infected with a microorganism or expressing a disease state.

10 30. The pharmaceutical composition of Claim 29 wherein the chemical bond is chemically cleaved inside a mammalian cell infected with a microorganism or expressing a disease state.

15 31. The pharmaceutical composition of Claim 29 wherein the chemical bond is a substrate for a protein having an enzymatic activity, said protein being specifically expressed in a mammalian cell infected with a microorganism or expressing a disease state.

Figure 1

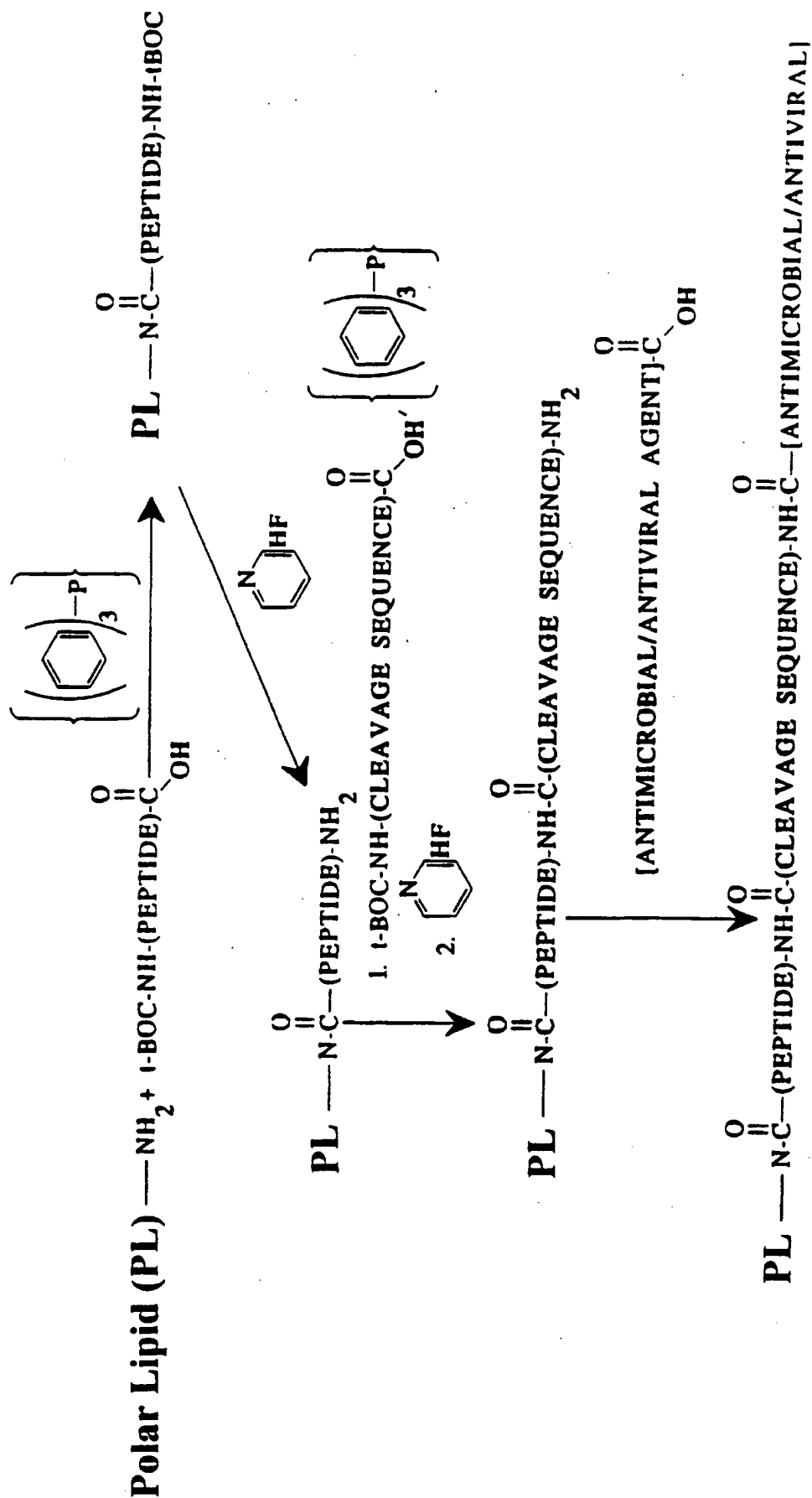


Figure 2

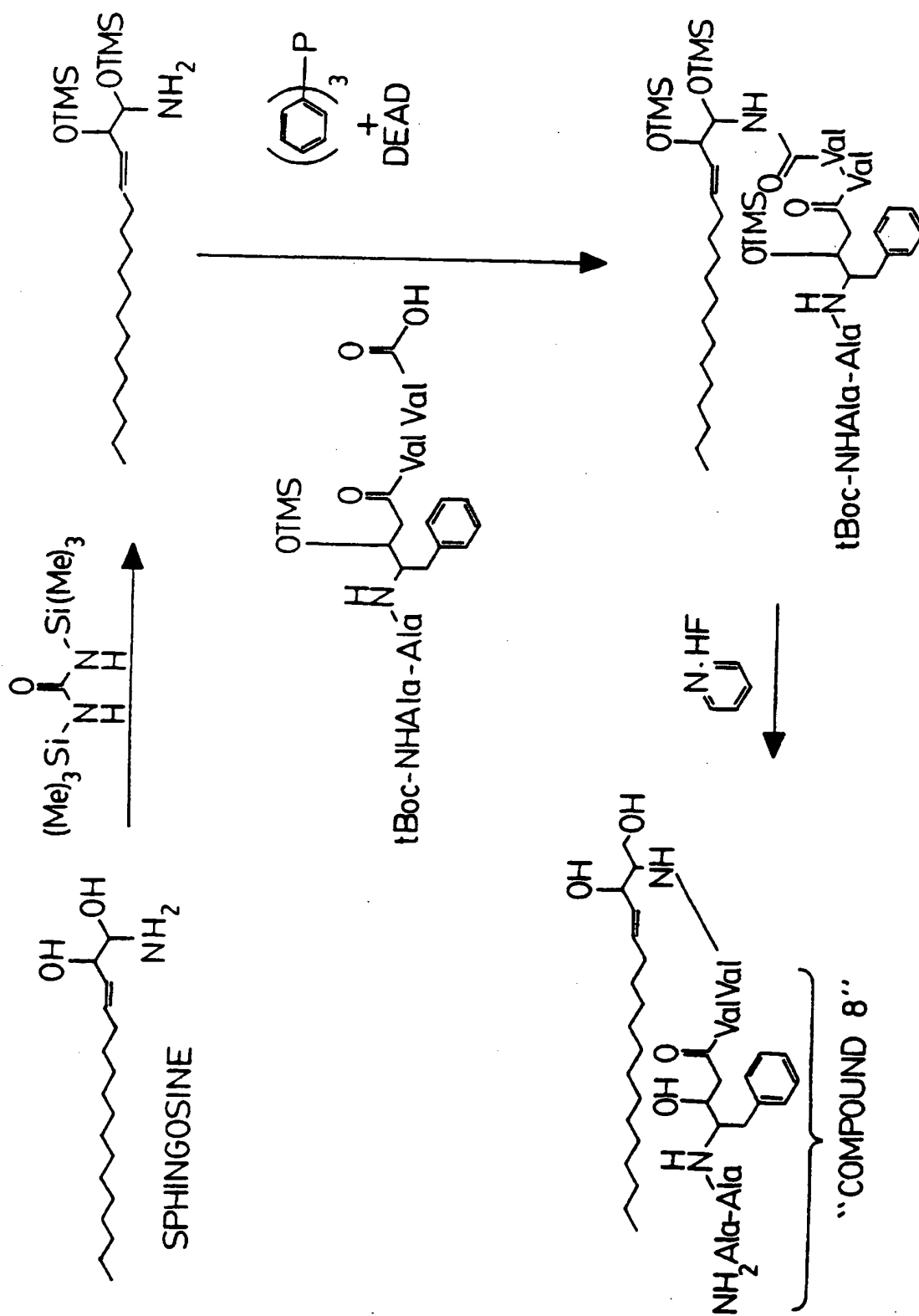


Figure 3

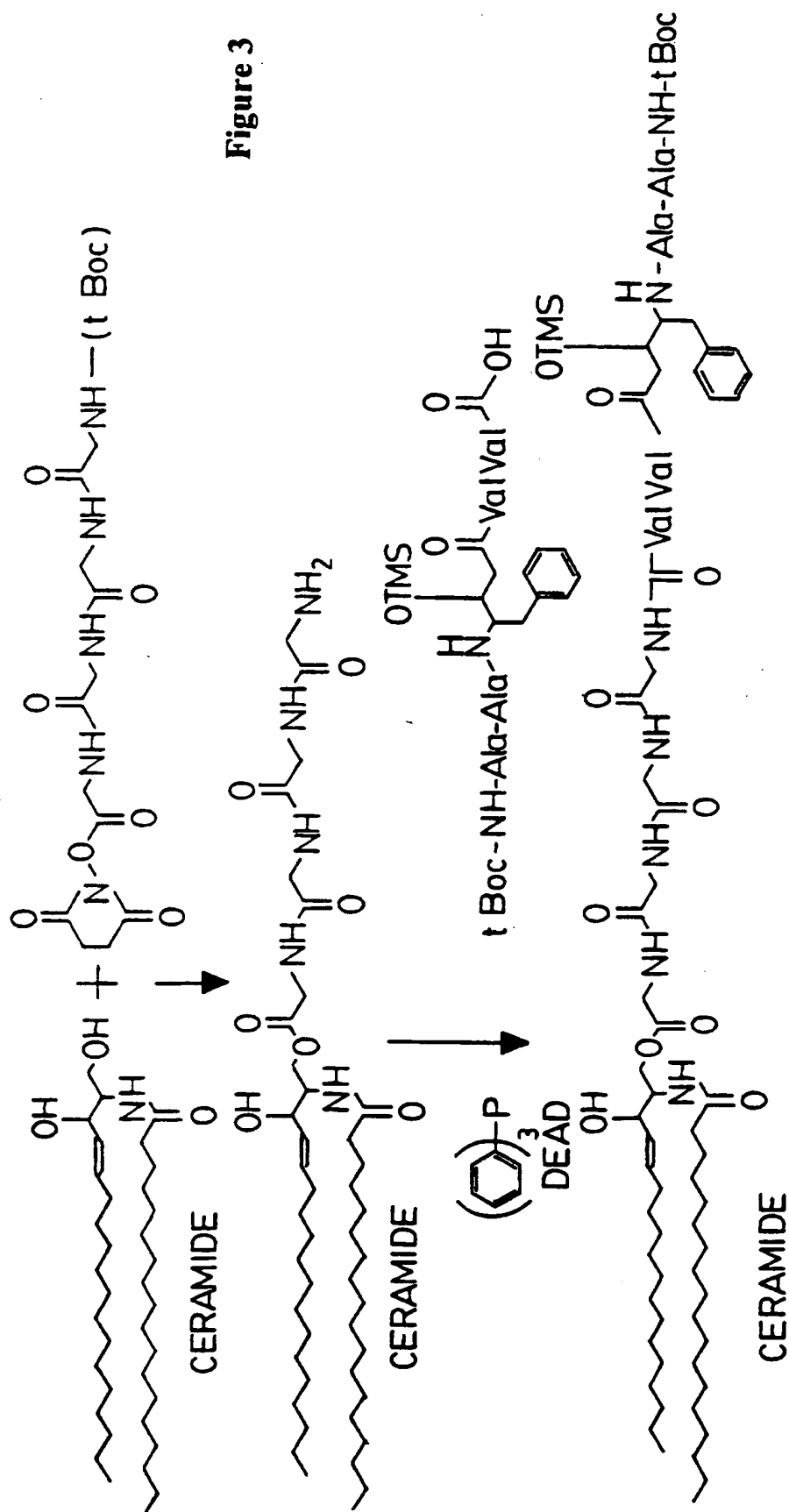
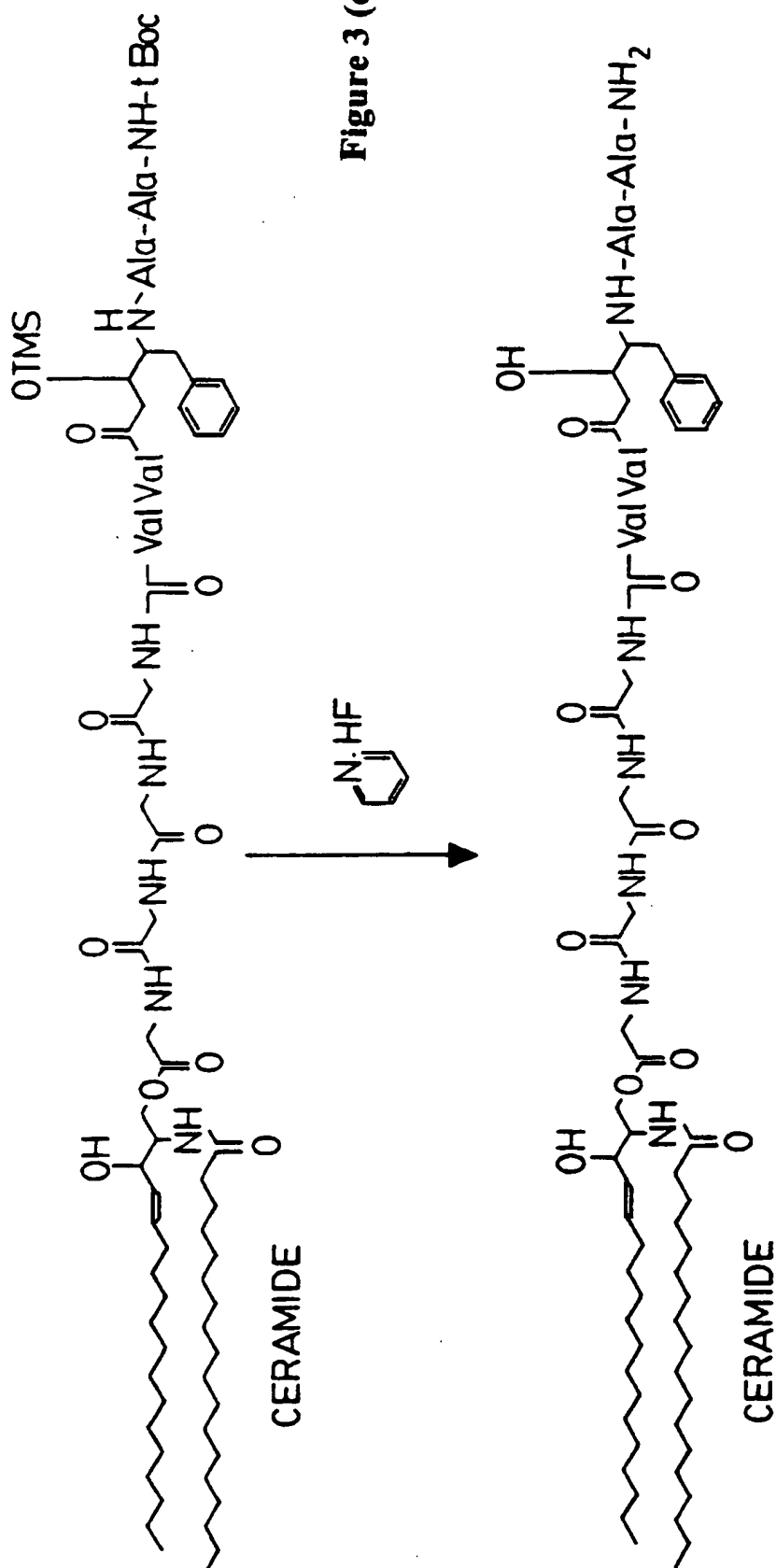
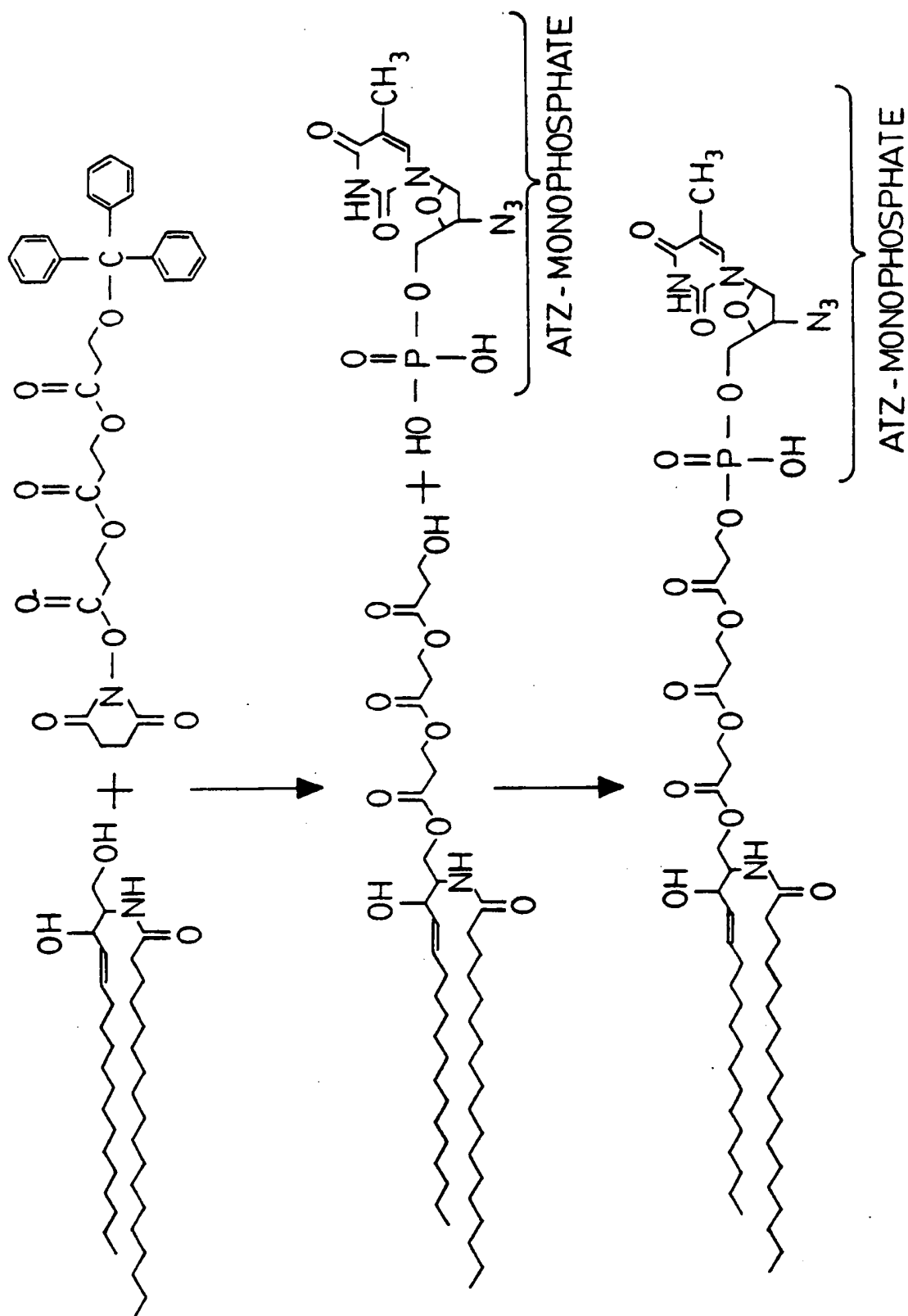


Figure 3 (cont'd.)





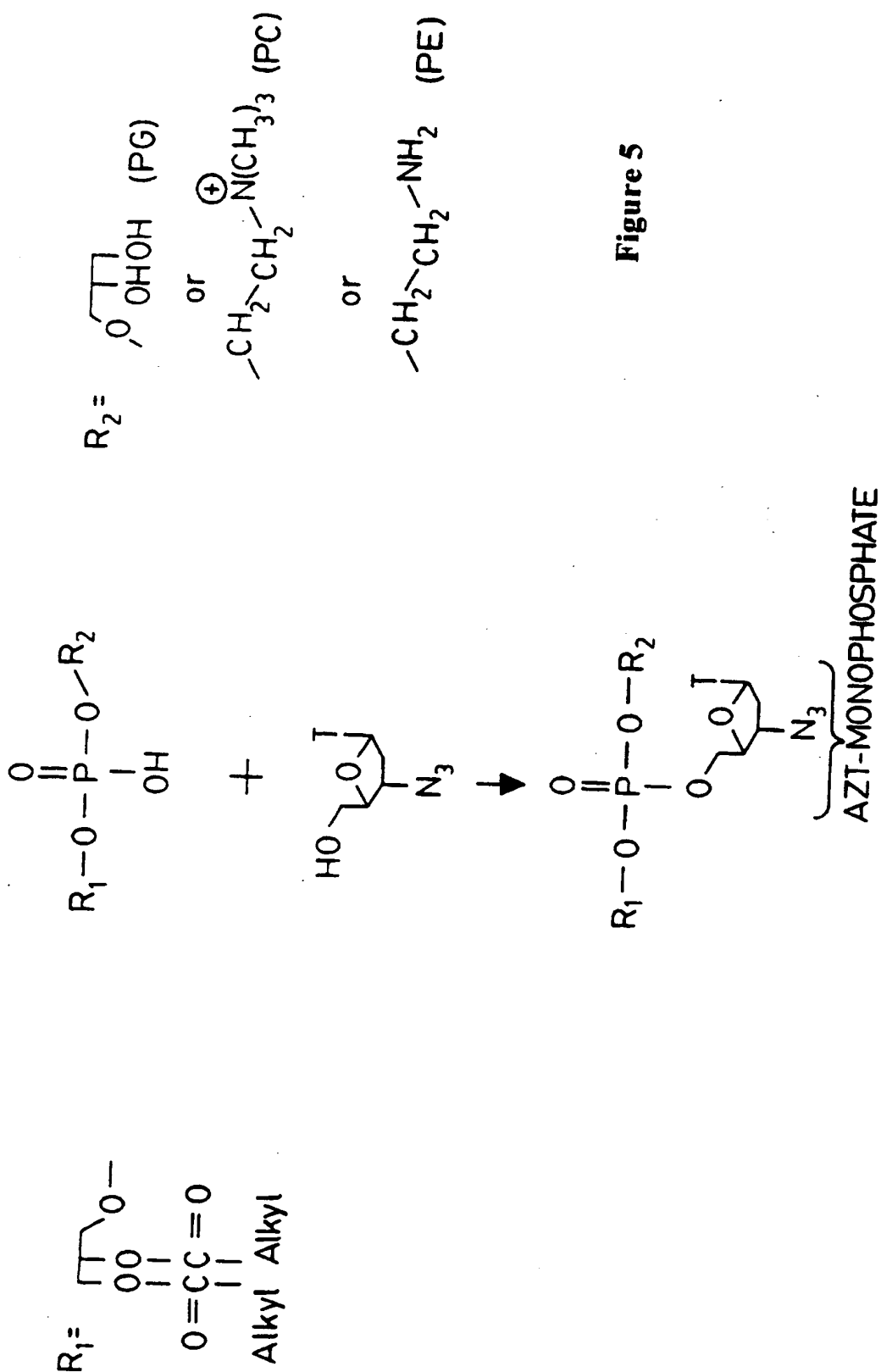
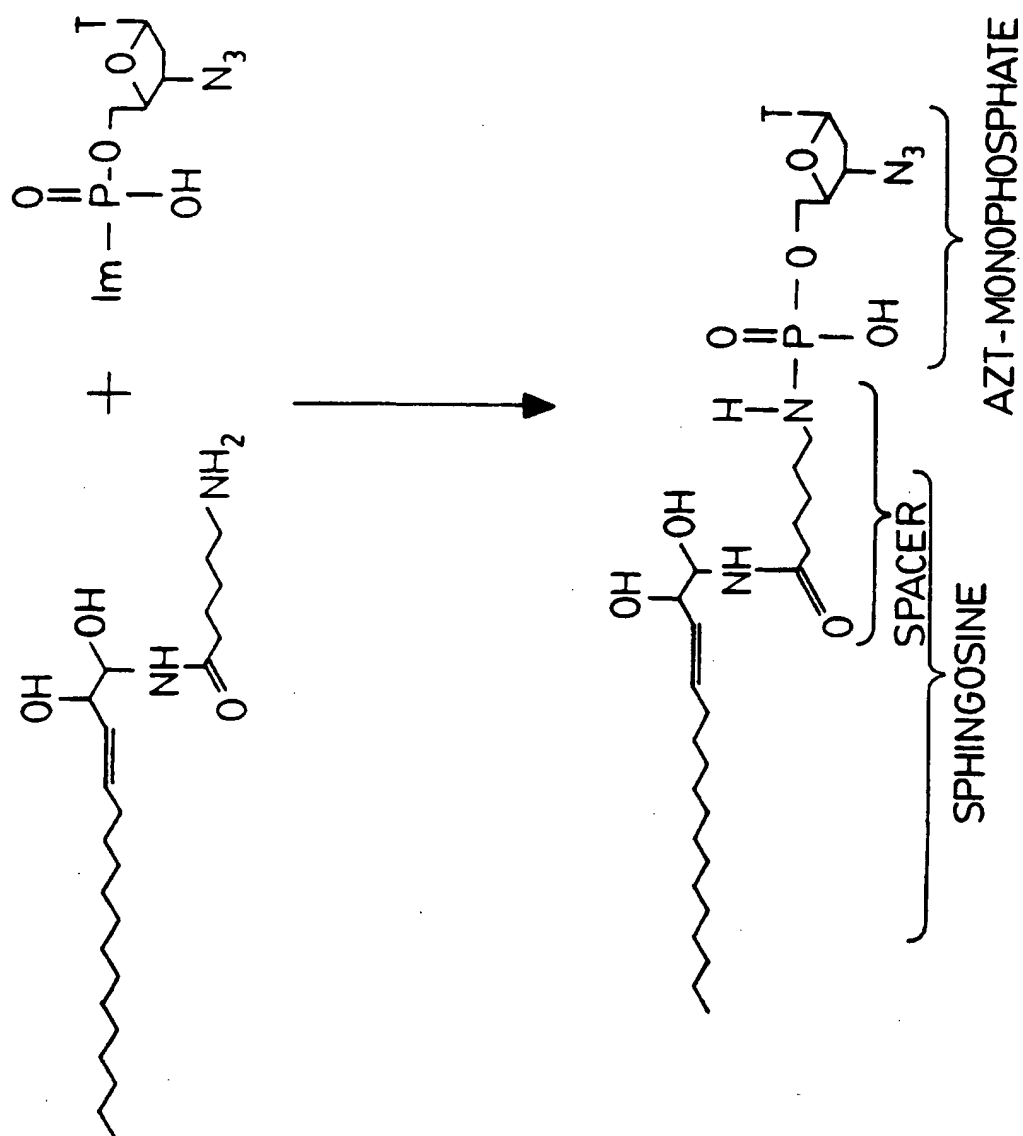


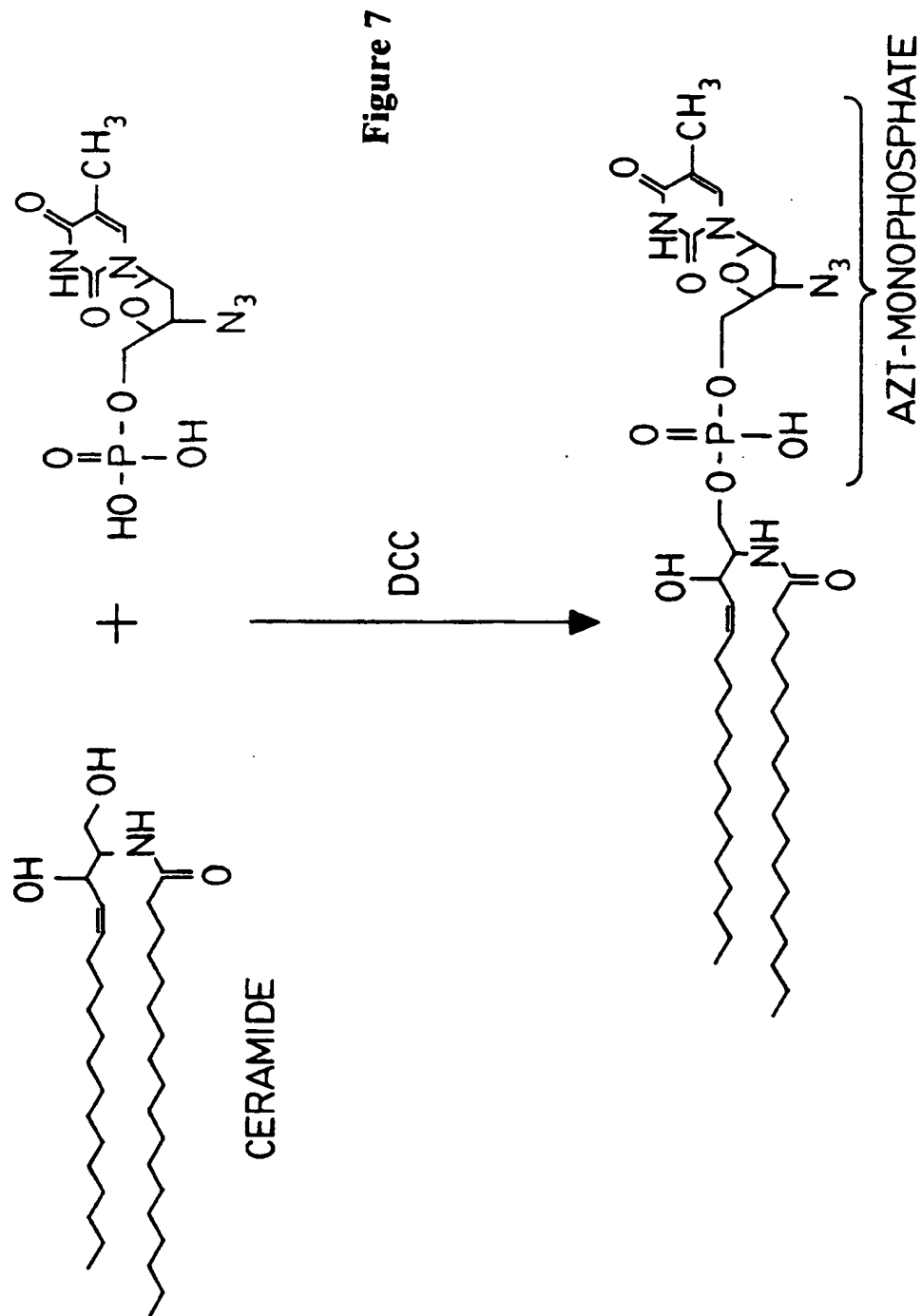
Figure 5

7/23

Figure 6

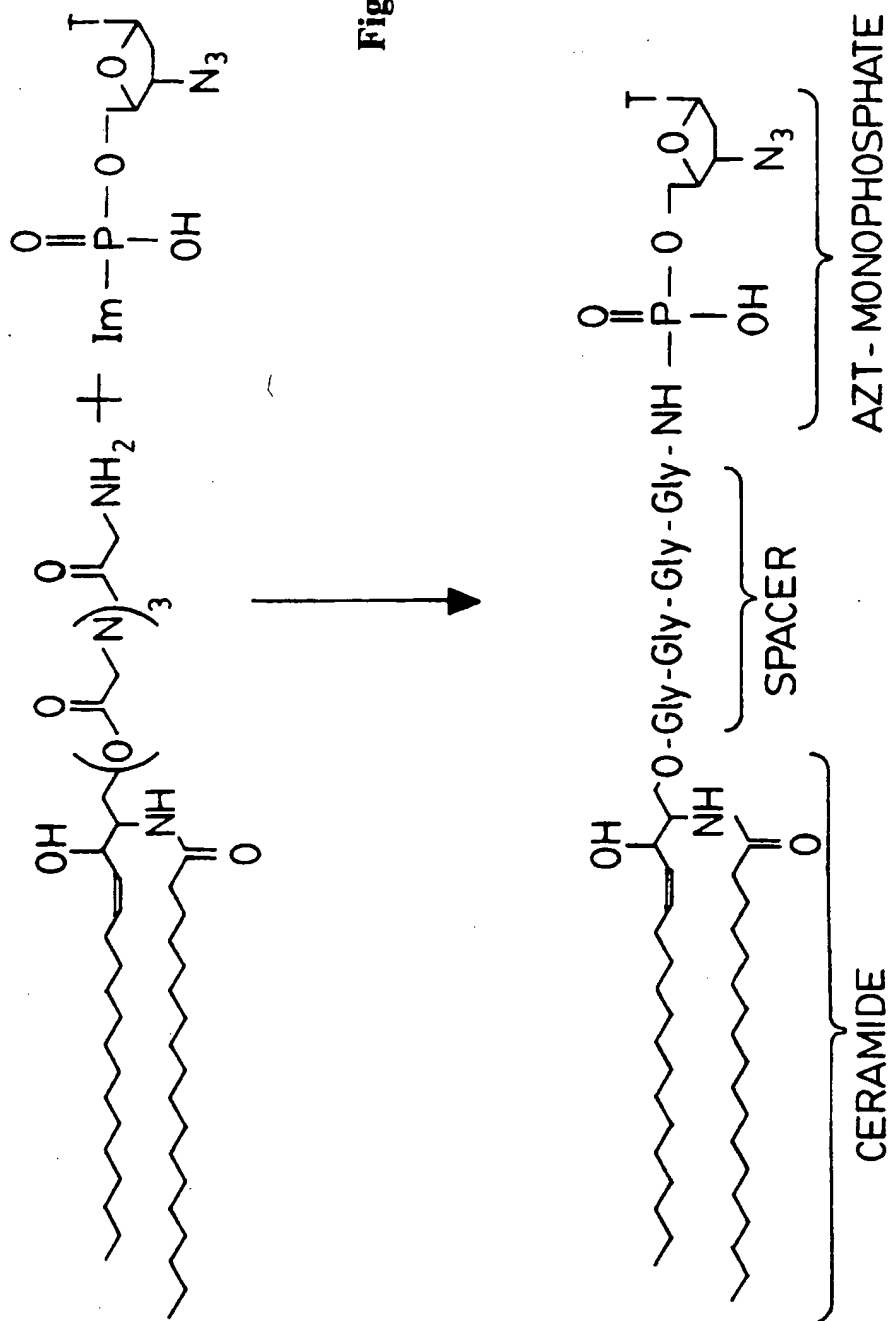


8/23



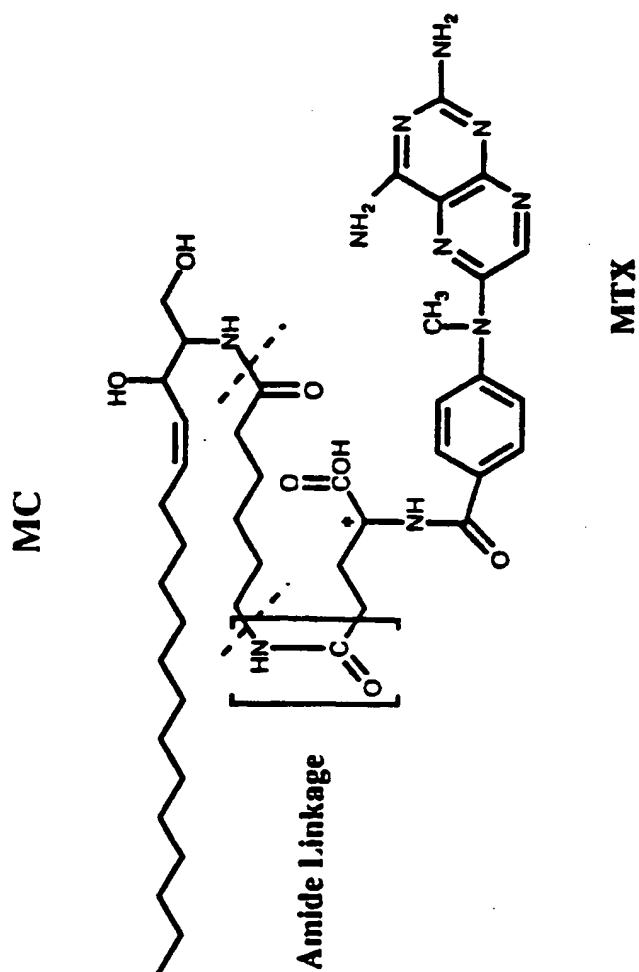
9/23

Figure 8



10/23

Figure 9A



11/23

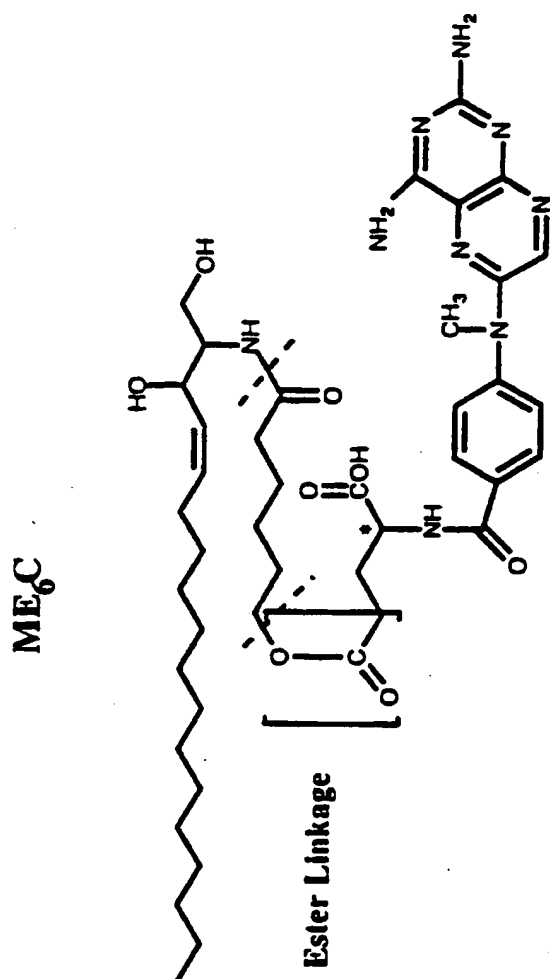


Figure 9B

12/23

MSC

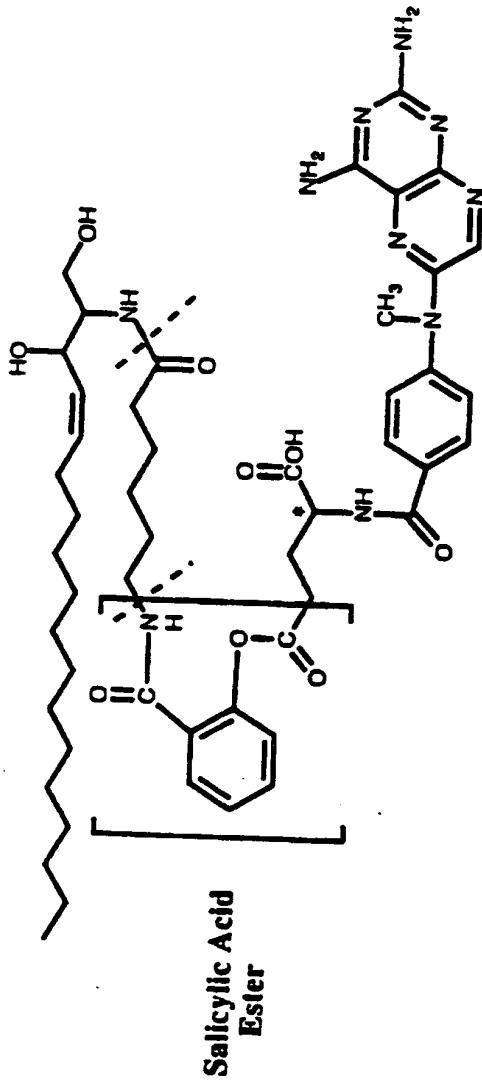


Figure 9C

MTX

13/23

AZT-Cer

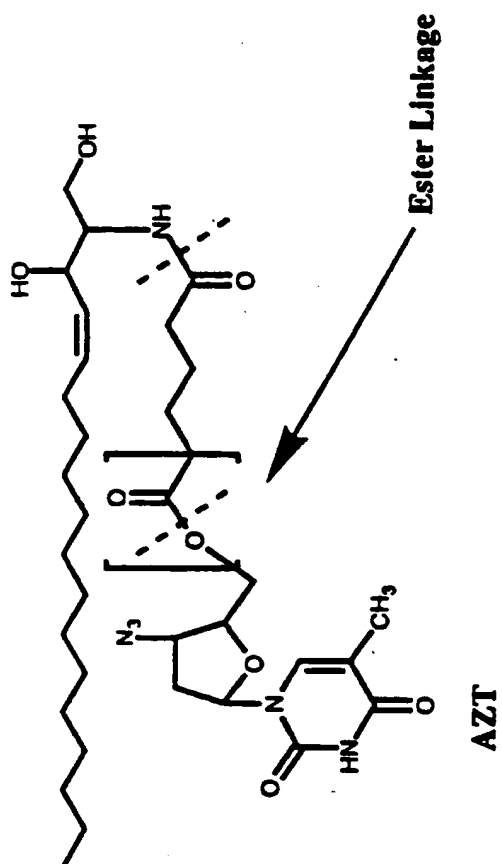


Figure 9D

14/23

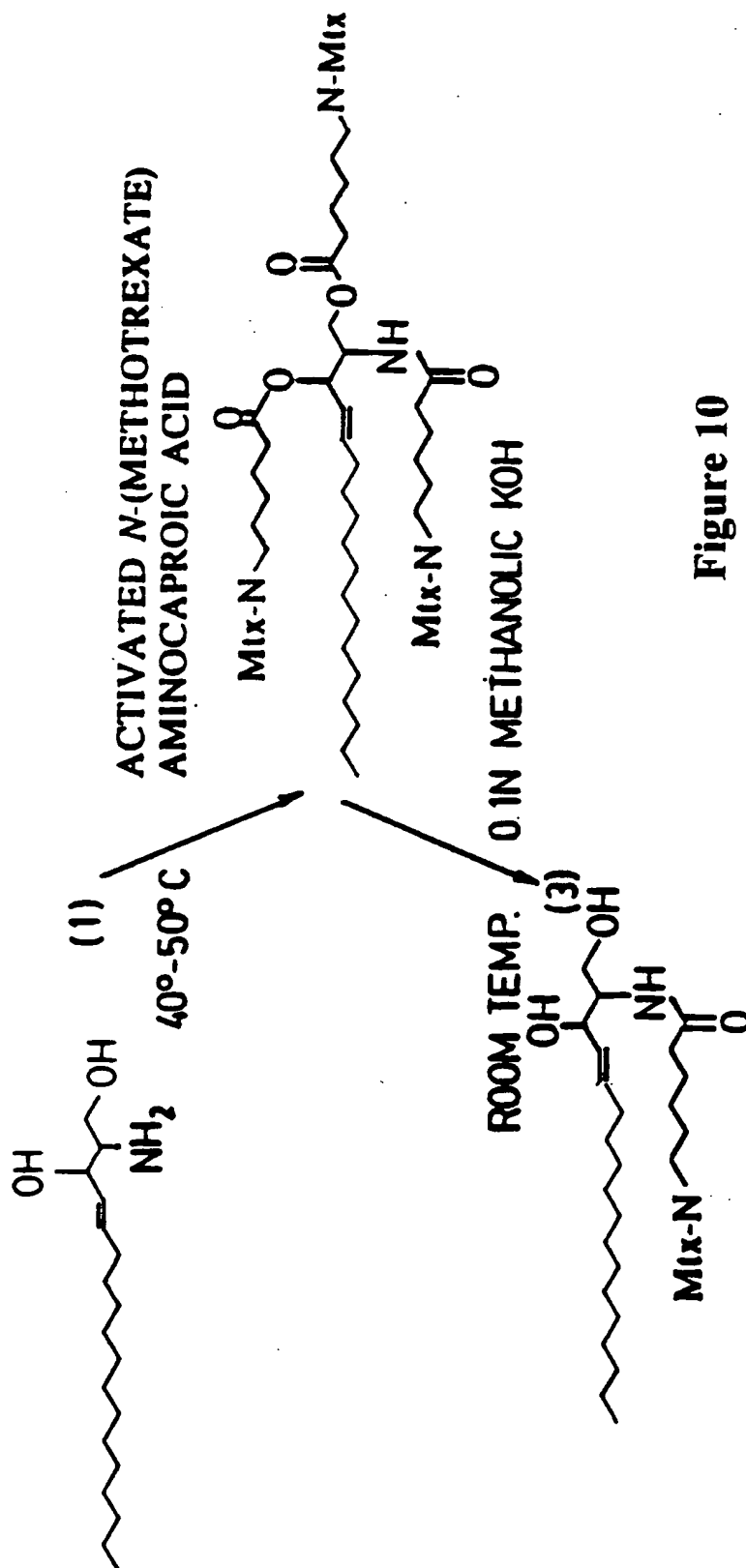


Figure 10

Figure 11



CORNIFIED LAYER →

Figure 12



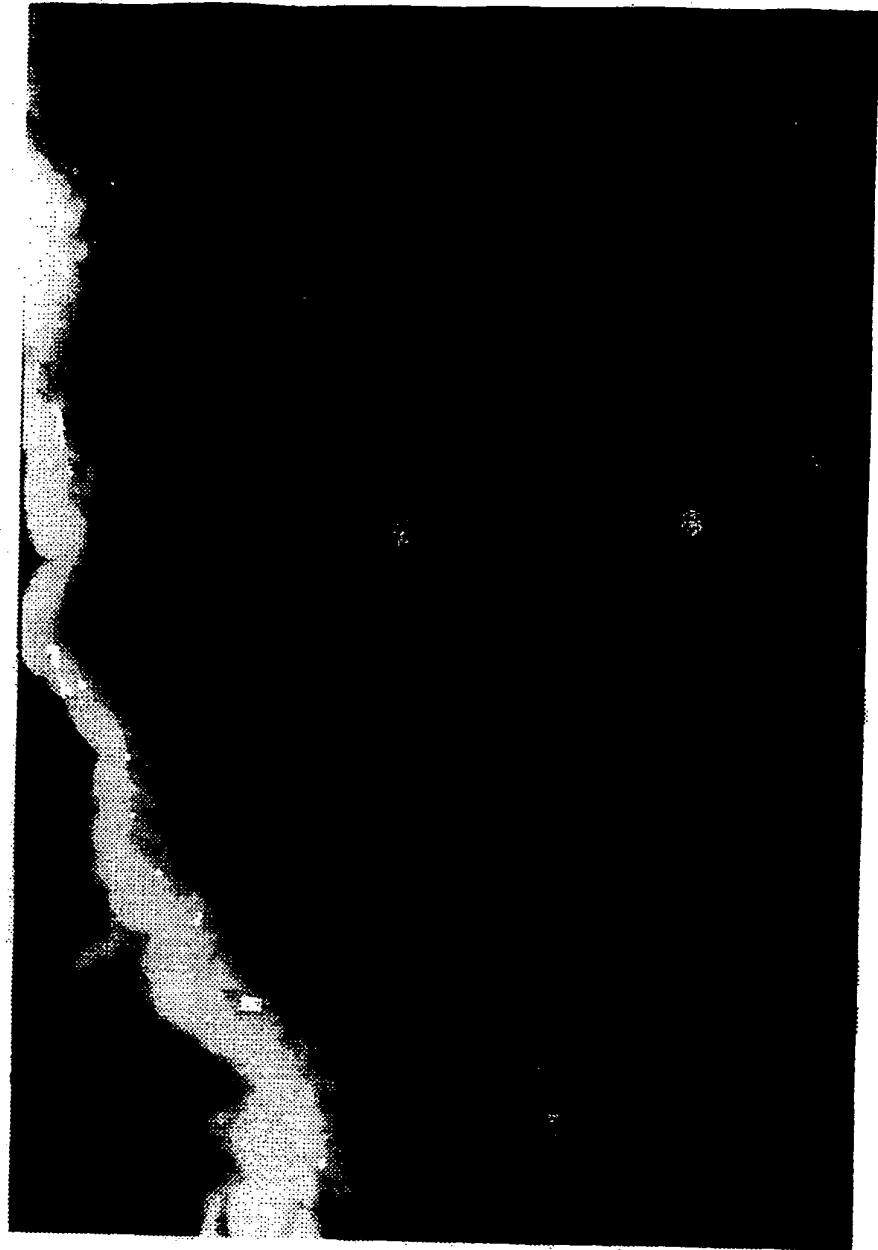
CORNIFIED LAYER →

Figure 13



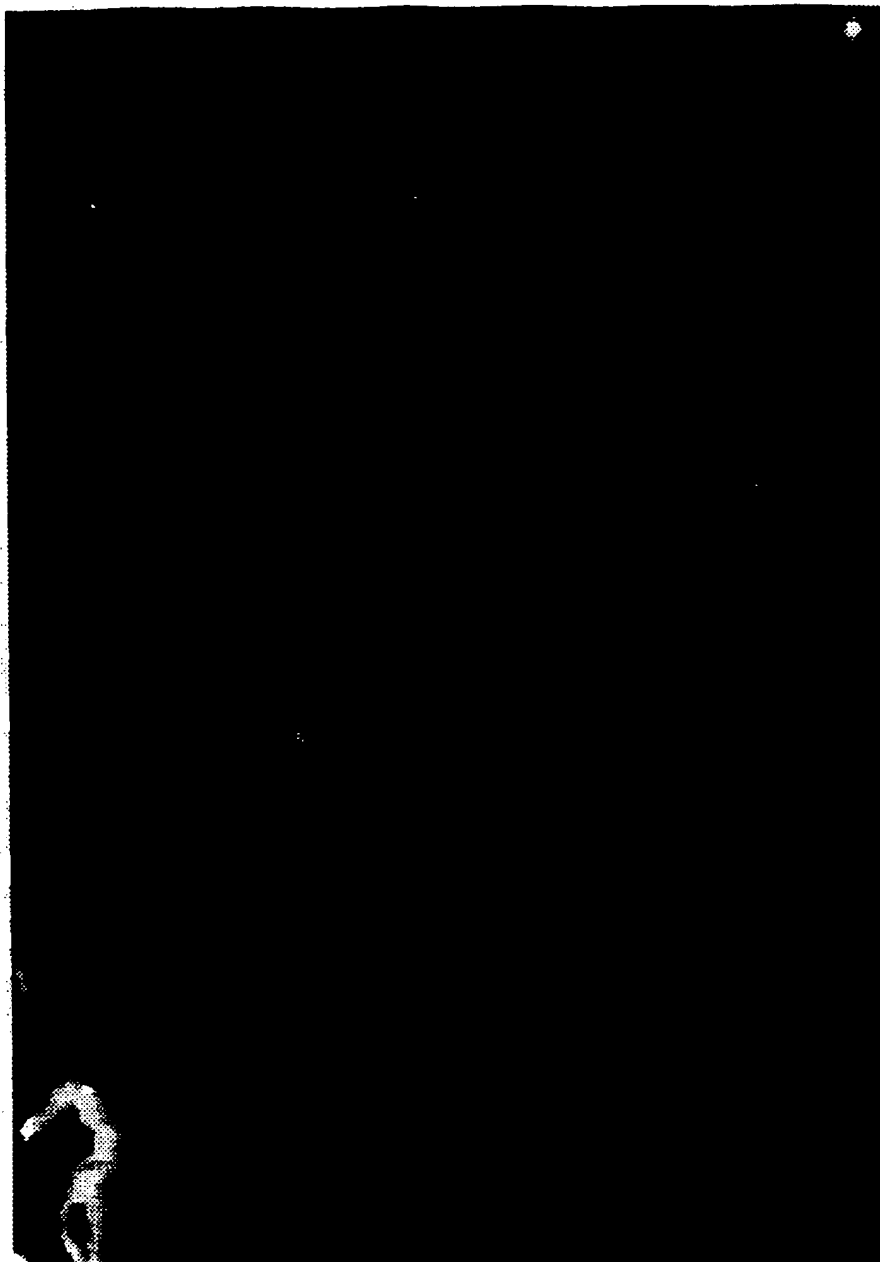
CORNIFIED LAYER →

Figure 14



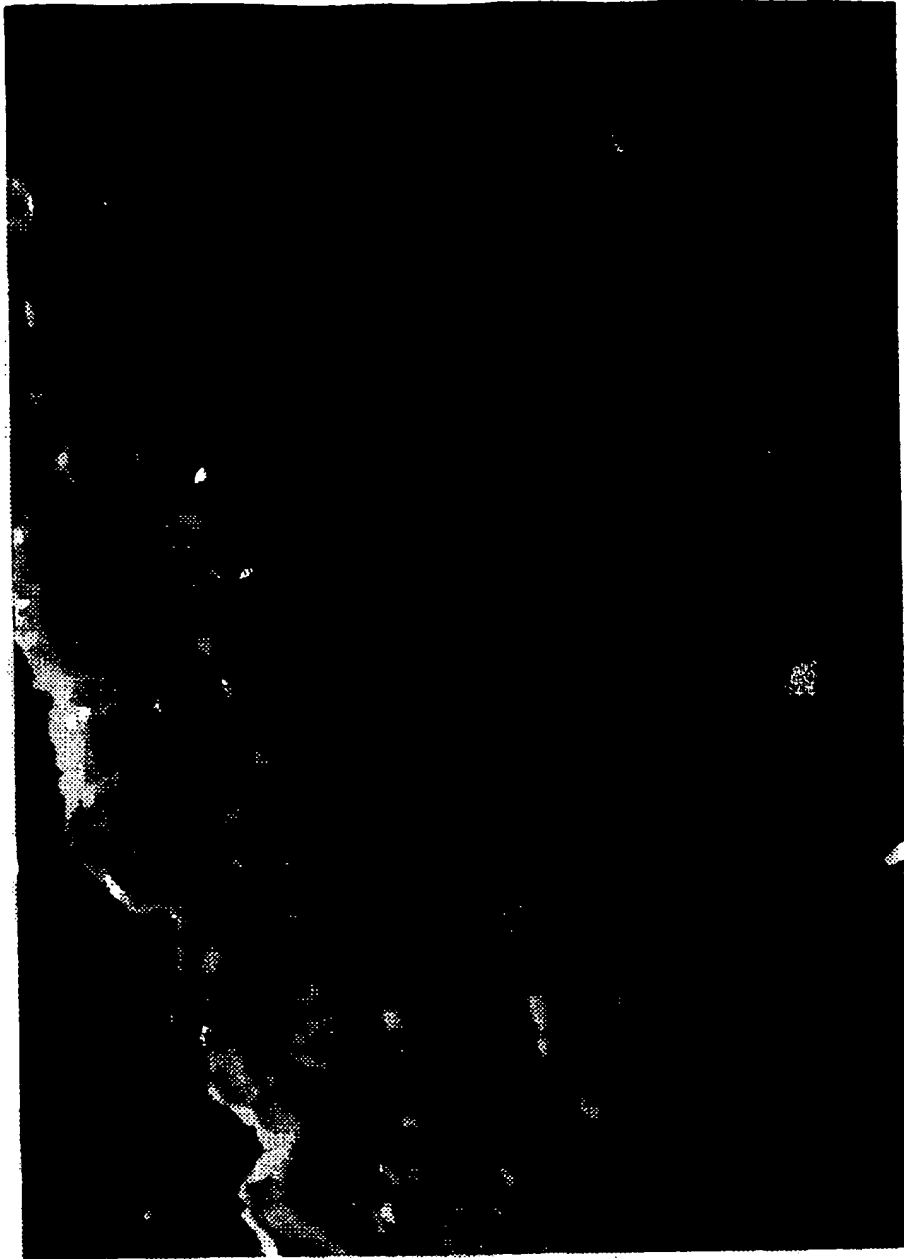
CORNIFIED LAYER →

Figure 15



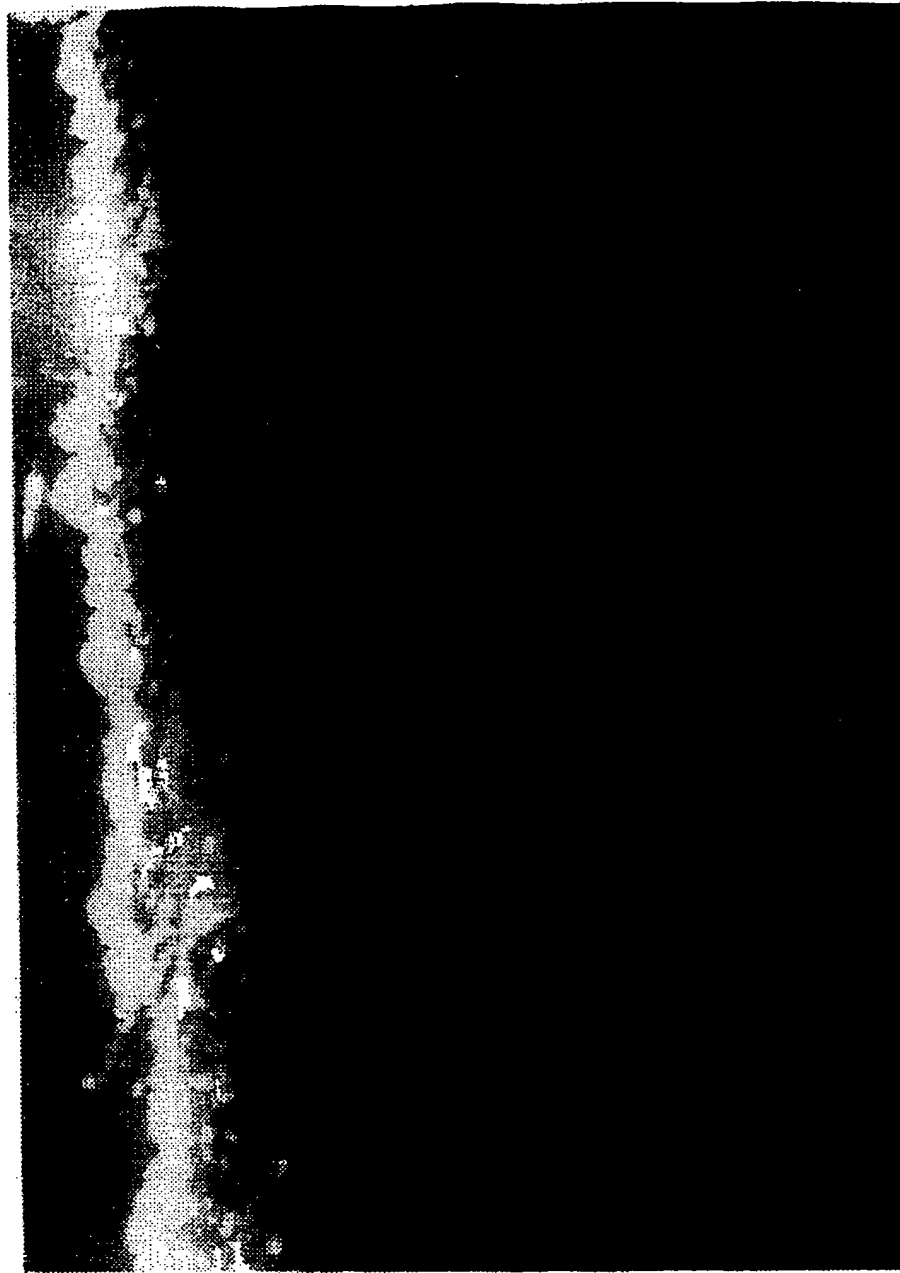
CORNIFIED LAYER →

Figure 16



CORNIFIED LAYER →

Figure 17



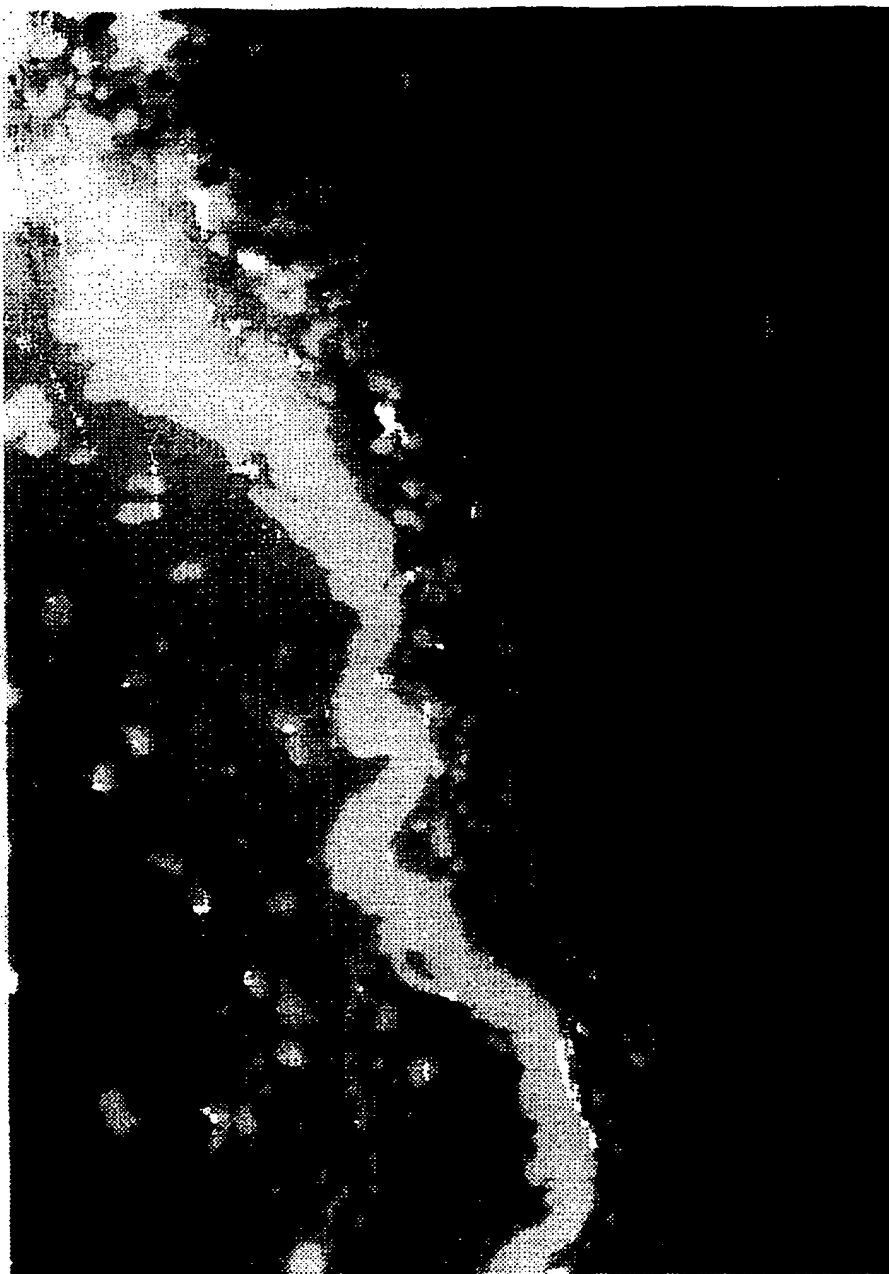
CORNIFIED LAYER →

Figure 18



CORNIFIED LAYER →

Figure 19



CORNIFIED LAYER →

INTERNATIONAL SEARCH REPORT

In: International Application No
PCT/US 96/12124

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 01138 A (OREGON STATE) 20 January 1994 see page 10, line 27 - page 11, line 7; claims; figures	1-31
X	WO 95 32002 A (OREGON STATE) 30 November 1995 see claims; figures 4,6; examples	1-31
X	US 5 149 794 A (YATVIN MILTON B ET AL) 22 September 1992 see claims; figures	1-31
X	WO 91 19726 A (UNIV WAKE FOREST ;UNIV NORTH CAROLINA (US)) 26 December 1991 see claims; figure 1	1-17, 26-31
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *A* document member of the same patent family

Date of the actual completion of the international search

10 April 1997

Date of mailing of the international search report

24.04.97

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Authorized officer

Berte, M

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/12124

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCONJUGATE CHEMISTRY, vol. 5, no. 2, 1 March 1994, pages 162-166, XP000434385 MENER F M ET AL: "SYNTHESIS OF A LIPID/PEPTIDE/DRUG CONJUGATE: N4-(ACYLPEPTIDYL)- ARA-C" see the whole document ---	1,6,7, 18,19, 22,26-31
X	W0 91 16024 A (VICAL INC) 31 October 1991 see page 20, line 29 - page 24, line 34 see page 14, line 3 - line 7 -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 96/12124

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9401138 A	20-01-94	US 5256641 A	26-10-93
		AU 4670093 A	31-01-94
		EP 0650371 A	03-05-95
		JP 7509227 T	12-10-95
		US 5543389 A	06-08-96
		US 5543390 A	06-08-96
		US 5543391 A	06-08-96
WO 9532002 A	30-11-95	US 5543390 A	06-08-96
		AU 2639395 A	18-12-95
		EP 0759784 A	05-03-97
		US 5543391 A	06-08-96
US 5149794 A	22-09-92	US 5543389 A	06-08-96
		US 5543390 A	06-08-96
		US 5543391 A	06-08-96
		US 5256641 A	26-10-93
WO 9119726 A	26-12-91	AT 137242 T	15-05-96
		AU 660417 B	29-06-95
		AU 8059791 A	07-01-92
		CA 2085354 A	16-12-91
		DE 69119074 D	30-05-96
		DE 69119074 T	12-12-96
		EP 0533825 A	31-03-93
WO 9116024 A	31-10-91	US 5264618 A	23-11-93
		AU 7854791 A	11-11-91
		EP 0523189 A	20-01-93
		JP 2538474 B	25-09-96
		US 5459127 A	17-10-95